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Prospecção de antibióticos e pigmentos a partir de bactérias isoladas da Antártica

Microbial prospecting for antibiotics and pigments from bacteria isolated from Antarctica.

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do Título de Doutor em Genética e Biologia Molecular, na área de Microbiologia

Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Ph.D in Genetics and Molecular Biology, in Microbiology field

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RESUMO

O continente Antártico é considerado uma região com condições climáticas extremas, com baixas temperaturas, alta incidência de radiação UV e déficit hídrico. Possui um bioma muito singular, o que significa que grande parte do seu patrimônio biológico não pode ser encontrada em outro lugar do planeta. Nestas condições espera-se encontrar micro-organismos adaptados (extremófilos) que possam ser explorados para a identificação de arsenais metabólicos únicos e versáteis com possíveis aplicações biotecnológicas em diversas áreas. O desenvolvimento de pesquisas recentes em nosso laboratório com bactérias e fungos da Antártica permitiu o isolamento de um grande número (em torno de 1200) de micro-organismos daquela região, muitos deles possivelmente indígenas, ainda não catalogados e não estudados quanto à produção de compostos bioativos potenciais. Com base nesse repertório, este estudo visou identificar as bactérias isoladas e descobrir produtos naturais de interesse biotecnológico, com foco em antibióticos e pigmentos para aplicação na indústria farmacêutica ou cosmética. Assim, um total de 326 isolados bacterianos, distribuídos em 39 gêneros diferentes, foram identificados com base no seqüenciamento do gene RNAr 16S. A triagem para antimicrobianos revelou quinze isolados capazes de inibir o crescimento de pelo menos uma das linhagens indicadoras: *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus subtilis* e *Candida albicans*. Uma bactéria psicrotolerante, *Pseudomonas* sp. isolado 99, apresentou amplo espectro antimicrobiano, além de atividade antiproliferativa e antiparasitária. Em relação aos pigmentos, foram selecionadas quatro bactérias que melhor resistiram à radiação UV e seus pigmentos foram extraídos e identificados. Todos os pigmentos selecionados foram identificados como carotenoides e apresentaram alta atividade antioxidante e boa estabilidade sob exposição à luz ultravioleta. Um pigmento em específico, identificado como all-trans-bacterioruberina, se mostrou seguro nos testes de fototoxicidade, abrindo perspectivas de uso na formulação de cosméticos.

Palavras-chave: Microbiologia Antártica, Pigmentos, Carotenoides, Antimicrobiano; Produtos Naturais.

ABSTRACT

The Antarctic continent is considered a region with extreme climatic conditions, with low temperatures, high incidence of UV radiation and water deficit. It has a very unique biome, which means that much of its biological heritage can not be found elsewhere on the planet. Under these conditions it is expected to find adapted microorganisms (extremophiles) that can be exploited for the identification of unique and versatile metabolic arsenals with potential biotechnological applications in several areas. The development of recent research in our laboratory with bacteria and fungi in Antarctica allowed the isolation of a large number (around 1200) of microorganisms, many of them possibly indigenous, not yet cataloged or studied for the production of potential bioactive compounds. Based on this repertoire, this study aimed to identify bacterial isolates and discover natural products of biotechnological interest, focusing on antibiotics and pigments for application in the pharmaceutical or cosmetic industry. Thus, a total of 326 bacterial isolates, distributed in 39 different genera, were recovered and identified based on 16S rRNA gene sequencing. Antimicrobial screening revealed fifteen isolates capable of inhibiting the growth of at least one of the indicator strains *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus subtilis* and *Candida albicans*. A psychrotolerant bacterium, *Pseudomonas* sp. isolate 99, showed broad antimicrobial spectrum, besides antiproliferative and antiparasitic activity. Regarding pigments, four bacteria were shown to better withstand UV radiation and their pigments were extracted and chemically identified. All selected pigments were identified as carotenoids and exhibited high antioxidant activity and good stability under ultraviolet light exposure. A specific pigment, identified as all-trans-bacterioruberina, proved to be safe in the phototoxicity tests, opening perspectives for further use in the formulation of cosmetics.

Keywords: Antarctic Microbiology; Pigments; Carotenoids; Antimicrobial; Natural products.

Sumário

INTRODUÇÃO

A biotecnologia de ambientes extremos é um campo emergente que engloba a biomedicina marinha (descoberta de novos fármacos), tecnologia de materiais, biorremediação, genética molecular, genômica, bioinformática, entre outras áreas. O interesse inicial para esta pesquisa é derivado da enorme biodiversidade e singularidade genética da vida no mar e no continente antártico. Muitas das formas de vida que residem no continente gelado são pouco conhecidas e, em se tratando de microbiologia de ambientes extremos, ainda caminhamos a passos lentos. Existe, portanto, uma grande lacuna de conhecimento sobre como os micro-organismos sobrevivem e reproduzem em condições tão adversas. A possibilidade de descobrirmos novos metabólitos para o benefício da população humana torna a pesquisa neste ambiente ainda mais fascinante e promissora.

Assim, algumas perguntas nortearam o desenvolvimento dessa tese:

- Qual o potencial da microbiota de bactérias da Antártica de produzir novos antibióticos?
- Estamos no caminho certo em procurar novos antibióticos em bactérias da Antártica?
- Quais são as novas formas de busca de produtos naturais oriundos de bactérias mundo afora?
- O que sabemos sobre pigmentos de bactérias da Antártica? Esses pigmentos teriam alguma aplicabilidade biotecnológica?

Com essas ideias em mente será apresentado a revisão da literatura e em seguida o desenvolvimento da tese na forma de capítulos.

REVISÃO DA LITERATURA

O Bioma Antártico

As condições ambientais no planeta Terra são extremamente diversificadas, com grandes variações de pressão, umidade, pH, temperatura e concentração de sal. Todos estes ambientes são habitados por organismos vivos, especialmente micro-organismos que se adaptaram em diferentes amplitudes de condições ambientais. Dentre estas, a baixa temperatura é muito comum tanto em ambientes naturais como artificiais. Cerca de 80% da biosfera e mais do que 90% dos ambientes marinhos têm temperatura inferior a 5°C (Margesin 2008). Ambientes com baixa temperatura são geralmente habitados por micro-organismos extremófilos, adaptados ao frio, com capacidade de crescer e sobreviver em condições adversas, tais como níveis de salinidade de 3,5%, às pressões que excedem 100 MPa, temperaturas extremamente flutuantes que podem atingir até -89°C, baixa disponibilidade de água e nutrientes e alta radiação solar (Kennedy et al. 2008; Kennedy et al. 2011). O continente Antártico se situa ao sul do círculo polar antártico e é considerado o mais frio, seco, com a maior média em altitude e o mais isolado de todos os continentes. Este isolamento provavelmente resultou na seleção/especiação de organismos incomuns, e ainda impediu a sua dispersão para outros continentes.

Um número crescente de estudos tem mostrado que existe uma grande diversidade microbiana nas regiões polares e diferentes tipos de atividades biológicas (Deming 2002; Bowman 2004; Chong et al. 2012; Tomova et al. 2013). Adaptações fisiológicas permitiram que os organismos psicrofílicos prosperassem nas regiões polares e, em especial, os micro-organismos são aparentemente os mais bem sucedidos. As explorações e investigações recentes de ambientes de baixa temperatura estão redefinindo os limites conhecidos da atividade microbiana.

Os limites funcionais do metabolismo das bactérias e da atividade das biomoléculas em baixas temperaturas permanecem mal definidos e pouco estudados. Individualmente, as biomoléculas parecem ser capazes de agir em baixas temperaturas. No entanto, os limites funcionais das biomoléculas dentro de sistemas

fisiológicos complexos podem variar drasticamente (Margesin et al. 2008). O limite para a reprodução de micro-organismos em baixas temperaturas é de -12°C, medido por turbidez de meio de cultura (Breezee et al. 2004), e a temperatura mínima para manter o metabolismo ativo é de -20°C, medido pela redução da resazurina em reações de respiração celular (Jakosky et al. 2003). Segundo esses estudos, a disponibilidade de água líquida parece ser o fator limitante de crescimento a temperaturas abaixo de zero.

Diversidade de bactérias na Antártica

A diversidade funcional das bactérias da Antártica pode ser caracterizada com base nas propriedades fisiológicas dos isolados. Bactérias **psicrofílicas** são definidas como organismos que podem crescer **a** ou **abaixo** de 0 °C, com temperaturas ótimas para crescimento menores que 15 °C e temperaturas máximas para crescimento menores que 20 °C; enquanto as **psicrotolerantes** podem crescer a 0 °C mas possuem temperatura de crescimento ótima acima de 15°C e temperaturas máximas para o crescimento acima de 20 °C (Ruisi et al. 2007; Moyer and Morita 2007; Clarke et al. 2013). Muitas são oligotróficas e pigmentadas (Margesin et al. 2008). Outras possuem propriedades metabólicas versáteis, como alta resistência ao estresse do meio ambiente, incluindo longos períodos de congelamento; ciclos de congelamento e descongelamento, dessecação, radiação solar, etc (Margesin et al. 2008).

Segundo Bowman et al. (1997), isolados bacterianos de gelo do mar da Antártica foram afiliados aos grupos: alfa e gama-proteobacterias, às bactérias Gram-positivas (Firmicutes e Actinobacterias), e ao grupo Flexibacter-Bacteroides-Cytophaga. A maioria dos isolados examinados parecia ser composta por novas espécies com base em comparações filogenéticas, com 45% das linhagens sendo psicrofílica. O sequenciamento do gene RNAr 16S revelou que os isolados psicrofílicos pertenciam aos gêneros *Colwellia*, *Shewanella*, *Marinobacter* e *Planococcus*, bem como a novas linhagens filogenéticas adjacentes a *Colwellia* e *Alteromonas*. Dentre as linhagens psicrofílicas, aquelas que se reproduzem em temperaturas abaixo de 7 °C (segundo “The Food Science Department at Cornell University”), foram encontrados membros dos gêneros *Pseudoalteromonas,*

Flexibacter, Bacteroides, Cytophaga, *Psychrobacter, Halomonas, Pseudomonas, Hyphomonas, Sphingomonas, Arthrobacter, Planococcus* e *Halobacillus*.

No trabalho de Van Trappen et al. (2002), foi estudada a diversidade de 746 bactérias heterotróficas isoladas de 10 lagos antárticos. Dessas bactérias, 40 linhagens diferentes foram identificadas, sendo elas pertencentes às classes Alfa, Beta e Gama-Proteobacteria, ao grupo das Gram-positivas com alta e baixa porcentagem G + C, e ao ramo Cytophaga-Flavobacterium-Bacteroides, resultados semelhantes àqueles do trabalho de Bowman et al. (1997). Outras bactérias não afiliadas com qualquer espécie conhecida foram identificadas, sendo relacionadas com *Alteromonas, Bacillus, Clavibacter, Cyclobacterium, Flavobacterium, Marinobacter, Mesorhizobium, Microbacterium, Pseudomonas, Saligentibacter, Sphingomonas* e *Sulfitobacter.*

Mais recentemente, uma nova empreitada realizada por pesquisadores belgas para se fazer o levantamento da diversidade bacteriana resultou no trabalho "Diversidade Microbiológica da Antártica - AMBIO" (Wilmotte et al. 2012), o qual foi baseado na coleta de amostras em nove pontos diferentes do continente antártico. Dessas amostras foram isolados em torno de 1.400 micro-organismos. A diversidade recuperada pertencia aos mesmos quatro principais filos descritos por Bowman et al. (1997): Actinobacteria, Bacteroidetes, Proteobacteria e Firmicutes. Os pesquisadores observaram ainda, com base na comparação com sequências de bases de dados públicas, que 42,2% das espécies recuperadas pareciam se restringir à Antártica (Wilmotte et al. 2012).

Várias novas espécies de bactérias foram isoladas do continente antártico nas últimas décadas, como, por exemplo, *Flavobacterium tegetincola* (McCammon e Bowman 2000), *Arthrobacter flavus* (Reddy et al. 2000), *Rhodoferax antarcticus* (Madigan et al. 2000), e os anaeróbios *Psychromonas antarcticus* (Mountfort et al. 1998) e *Clostridium vincentii* (Mountfort et al. 1997).

Micro-organismos da Antártica: uma fonte inexplorada de novas moléculas

Do ponto de vista histórico, micro-organismos cultiváveis do solo têm sido uma fonte inestimável para a produção de compostos naturais com atividades biológicas importantes para a humanidade. (Bull et al. 2000; Zhang and Demain 2005; Brötz-Oesterhelt and Sass 2010). Durante os últimos 50 anos, produtos derivados de metabólitos secundários têm sido usados nas áreas médica, industrial e agrícola, como os antibióticos, drogas anticarcinogênicas, antifúngicas, agentes imunossupressores, enzimas e polímeros para aplicações industriais e tecnológicas, herbicidas, inseticidas, promotores de crescimento, entre outros. Outras enzimas microbianas são importantes para pesquisa acadêmica e desenvolvimento de aplicações biotecnológicas, incluindo as enzimas de restrição e polimerases de ácidos nucleicos, as quais são rotineiramente utilizadas na tecnologia do DNA recombinante (de Miguel Bouzas et al. 2006; Cavicchioli et al. 2011; Loenen et al. 2014).

Tradicionalmente, compostos com atividade biológica têm sido acessados por métodos envolvendo isolamento e cultivo dos organismos a partir das amostras ambientais, principalmente do solo. Isolados e/ou produtos de fermentação passam por um processo de triagem para as atividades desejadas.

Assim como acontece com os ambientes aquáticos, terrestres e outros, onde se sabe que mais de 99% das bactérias não podem ser cultivadas por meios convencionais, a maioria dos micro-organismos da Antártica ainda não foi cultivada ou mesmo identificada (Kennedy et al. 2010; Kennedy et al. 2011). Estes dados sugerem que a grande maioria da diversidade microbiana neste ecossistema ainda permanece inexplorada, oferecendo uma fonte inestimável de novas famílias de moléculas, como antibióticos, biocatalisadores, entre outros (Kennedy et al. 2010; Kennedy et al. 2011).

Antibióticos: Histórico e Perspectivas

Antimicrobianos são, provavelmente, uma das formas mais bem sucedidas de fármacos da história da medicina. Não é necessário reiterarmos aqui quantas vidas eles têm salvado e como têm contribuído para o controle de doenças infecciosas, reduzindo as taxas de morbidade e mortalidade humana. Selman Waksman (Nobel de

medicina 1952 - descobridor da estreptomicina) usou pela primeira vez a palavra antibiótico para descrever qualquer molécula pequena produzida por um micro-organismo que antagoniza o crescimento de outros micróbios. No entanto, foi outro cientista, Alexander Fleming em 1928, que descobriu o primeiro antibiótico de uso comercial, oriundo de fungos, a penicilina. Hoje já contam mais de 12.000 tipos diferentes de antibióticos, sendo que 300 deles são comercializados (Demain 1999). Em uma lista rápida poderíamos citar, por exemplo, os seguintes antibióticos: β-Lactâmicos como Penicilinas, Cefalosporinas, Carbapenems e Monobactans; Quinolonas, Glicopeptídeos, Oxazolidinonas, Aminoglicosídeos, Macrolídeos, Lincosaminas, Nitroimidazólicos, Cloranfenicol, Estreptograminas, Sulfonamidas, Tetraciclinas, e os mais novos antimicrobianos Glicilciclinas, Polimixinas, Daptomicina e Gemifloxacina.

No entanto, a maioria dos antibióticos em circulação hoje em dia foi descoberta há seis décadas, durante a fase de ouro dos antibióticos (Aminov 2010). Após esse período, encontrar novos antibióticos se tornou cada vez mais difícil, muito dinheiro era despendido para minerar algo novo e pouco retorno era obtido. Além disso, existia a crença de que as doenças infecciosas da época seriam tratadas pelos antibióticos existentes no mercado. Assim, grandes indústrias farmacêuticas viram seus lucros diminuírem vertiginosamente, e reduziram o empenho em se prospectar novos antibióticos, enquanto elevavam os esforços com outras doenças ditas mais "rentáveis", como diabetes e asma (Handelsman 2005). Nos anos seguintes, até o final da década de 90, o que houve foi a reformulação e combinação entre diferentes antibióticos já existentes, para combater as novas infecções circulantes.

A partir dos anos 2.000 até os dias de hoje, poucos agentes antibacterianos com novos mecanismos de ação foram aprovados. A droga sintética *Linezolida*, da classe das "oxazolidinonas", e a *Daptomicina*, lipopeptídeo isolado de produtos naturais (Spellberg et al. 2007; Leach et al. 2011) são exemplos de novos antibióticos recém descobertos. A *Linezolida* é tratada como super-antibiótico e só é utilizada em casos extremos de resistência bacteriana a outras drogas. Teve um custo de 500 milhões de dólares para ser sintetizada. Seu mecanismo de ação consiste no bloqueio da síntese proteica, pois se fixa na unidade 50S do ribossomo (Swaney et al. 1998). A *Daptomicina* é um antimicrobiano lipopeptídico cíclico, obtido da fermentação da

bactéria *Streptomyces roseosporus*. Seu mecanismo de ação envolve a desorganização de múltiplas funções da membrana celular bacteriana (Pogliano et al. 2012).

Acontece que apesar dos grandes esforços na busca de novos antibióticos, bactérias estão ficando resistentes mais rapidamente do que a chegada de novas drogas ao mercado. A evolução da resistência aos antibióticos por importantes patógenos humanos tornou aqueles primeiros antibióticos, e a maioria de seus sucessores, em grande parte ineficazes (Spellberg et al. 2004; Spellberg et al. 2013). De acordo com o mesmo autor, "os riscos para a saúde humana vêm sob a forma de resistência das bactérias aos antibióticos. Vivemos em um mundo bacteriano onde nós nunca seremos capazes de ficar à frente de suas curvas de mutação. Um teste de nossa resiliência é o quão distante da curva nos deixaremos ficar para trás".

Uma coisa é certa, quanto maior o uso de antibióticos, maior a pressão seletiva sobre as bactérias, ou seja, maior é a seleção de bactérias resistentes a estes. Mas não é apenas dessa forma que se originam as resistências. Recentemente, foram descobertas bactérias resistentes a antibióticos em cavernas subterrâneas, geologicamente isoladas da superfície do planeta por quatro milhões de anos (Bhullar et al. 2012). Notavelmente, a resistência foi encontrada até a antibióticos sintéticos que não existiam na Terra até o século XX. Estes resultados mostram uma crítica realidade: a resistência aos antibióticos existe e é amplamente disseminada na natureza, até mesmo a medicamentos que ainda não foram inventados.

Segundo artigo publicado pelo jornal "The Lancet", escrito por Teillant et al. (2015), estima-se que entre 38,7 e 50,9% dos patógenos causadores de infecção após intervenção cirúrgica e 26,8% após quimioterapias são resistentes aos antibióticos profiláticos padrão nos EUA.

Em outra manchete, Barack Obama pede mais dinheiro para o empenho na causa. "...o presidente dos EUA deseja duplicar os fundos em seu orçamento para 2016 para o combate às bactérias resistentes a antibióticos, indicou nesta terça-feira a Casa Branca": projeto de orçamento pedirá para esta causa US$ 1,2bilhão (fonte: Portal Terra, 2015. Acesso em: 15 ago 2016).

No Brasil, casos de infecções hospitalares por bactérias super-resistentes têm sido cada vez mais comuns. Relatos do Hospital Albert Eistein associam em torno de 18 mortes à super-bactéria KPC no Distrito Federal e mais 22 pessoas infectadas em

outros quatro estados (fonte: Portal Hospital Albert Einstein, 2014. Acesso em: 15 ago 2016).

Outras manchetes sobre bactérias multirresistentes são descritas abaixo*:*

- *Superbactérias matarão uma pessoa a cada 3 segundos em 2050* (Fonte: Portal UOL, 2015. Acesso em: 15 ago 2016).
- *Resistência à antibióticos uma 'ameaça global', a OMS adverte* (Fonte: Portal BBC News, 2014. Acesso em: 15 ago 2016).

A busca, portanto, por novos antibióticos é de grande importância, já que os atuais parecem não apresentar mais tanta eficácia aos patógenos emergentes. Recentemente, através de novas tecnologias de cultivo de micro-organismos ditos não cultiváveis, uma nova categoria de antibiótico foi descoberta. Neste artigo, publicado na *Nature*, o antimicrobiano isolado de bactérias da classe Betaproteobacteria, chamado de “Teixobactina”, seria capaz de agir contra as bactérias Gram-positivas multirresistentes a antibióticos (Ling et al. 2015).

Assim, uma alternativa na busca de novas drogas é explorar a biodiversidade em ambientes com características únicas, os quais abrigam micro-organismos com propriedades fisiológicas e metabólicas particulares, que lhes permitem adaptar e sobreviver em condições inóspitas. A prospecção da diversidade microbiana representa ainda uma promissora fonte para a obtenção de novas e diferentes moléculas antimicrobianas.

Para acessar essa diversidade é oportuno explorar novos ambientes, tais como ambientes extremos e pouco explorados como a Antártica, onde a quantidade de publicações na área ainda é muito baixa.

Lewis (2012) sugere que deveríamos reativar a plataforma usada por Waksman (descobridor da estreptomicna) para a descoberta de novos antibióticos. A estratégia consite em refazer testes de antibiose usados na década de 50 para a prospecção de antibióticos. Só que naquela época métodos convencionais de isolamento e ambientes comuns eram utilizados, e hoje novas estratégias e metodologias como o uso do iChip (Nichols et al. 2010; Ling et al. 2015) estão sendo empregadas para recuperar a diversidade presente na natureza. Isso pode auxiliar na busca por novos micro-

organismos, aumentando as chances de se acessar a diversidade química que era previamente perdida.

Metabólitos secundários da microbiota antártica

Metabólito secundário é um epíteto muito usado nas discussões sobre fisiologia microbiana, vegetal e bioquímica. Entende-se que estes compostos são característicos de grupos taxonômicos distintos de organismos, e têm em geral uma estrutura química complexa, diversificada e incomum. São compostos orgânicos que não estão diretamente envolvidos nos processos primários de crescimento, desenvolvimento e reprodução dos organismos (Fraenkel 1959).

Uma recente revisão elaborada por Cragg e Newman (2013), intitulada "*Natural products: A continuing source of novel drug leads*", que traduzida seria "Produtos naturais: Uma fonte contínua de pistas por novas drogas" traz um relato detalhado do histórico por trás do surgimento de novas drogas no mercado a partir de produtos naturais. Em um de seus capítulos, ele dedica especial atenção para os compostos produzidos por micro-organismos que foram descobertos nas últimas décadas. Dentre eles, podemos citar os antibacterianos, como a tetraciclina (de *Actinomycetales*) e cefalosporina (de *Cephalosporium acremonium*), agentes imunossupressivos, como ciclosporina (de espécies de *Trichoderma* e *Tolypocladium*) e a rapamicina (de *Streptomyces*), agentes antifúngicos, como a candicidina (de *Streptomyces griseus*) e nistatina (de *Streptomyces noursei*). Ainda, fármacos anticancerígenos, tais como antraciclina, bleomicina e mitomicina, todos isolados de espécies de *Streptomyces* (Crooke and Bradner 1976; Oki 1977; Hecht 2000), e o anti-helmíntico e anti-parasítico ivermectina [de *Streptomyces* – descoberta que levou o Nobel de medicina de 2015 (The Nobel Assembly at Karolinska Institutet et al. 2012).

Em micro-organismos, esses metabólitos são tipicamente produzidos após a fase de crescimento exponencial, quando eles já se encontram em fase estacionária. Sua gama de atividades biológicas é vasta, incluindo matar ou inibir o crescimento de outros micro-organismos, podendo também ser tóxico para os organismos multicelulares, como invertebrados e plantas (Demain and Fang 2000). Podem apresentar, também, papéis semelhantes a hormônios na diferenciação microbiana.

Muitos desses compostos estão relacionados com alguma vantagem competitiva para o produtor (O'Brien et al. 2004).

Tendo em conta as condições ambientais severas prevalentes na Antártica, tem-se argumentado que a produção de metabólitos secundários, como os compostos antimicrobianos extracelulares, seriam uma vantagem particular na redução da competição interespécies. Portanto, foi sugerido que as regiões polares podem ser vistas como um vasto reservatório inexplorado de potenciais micro-organismos produtores de antibióticos (O'Brien et al. 2004).

Em relação aos antimicrobianos estudados da Antártica, os filos Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes e Cianobacteria possuem representantes com capacidade de produção desses compostos. Liu et al. (2013), em sua recente revisão sobre produtos naturais obtidos de organismos das regiões polares, listam uma série de compostos microbianos com atividade antibacteriana. Dentre eles, dois pigmentos arroxeados (violaceínas) da proteobacteria *Janthinobacterium* sp. (Mojib et al. 2010), que possuem uma concentração mínima inibitória (MIC) anti-*Mycobacterium tuberculosis* 15 vezes menor do que o pigmento das linhagens de *Chromobacterium violaceum* contra a mesma cepa. Outros autores também descreveram esta habilidade para outros representantes do filo Proteobacteria isolados da Antártica (Médigue et al. 2005; Rojas et al. 2009; Lee et al. 2012; Fukuda et al. 2013; Papa et al. 2013; Asencio et al. 2014; Tomova et al. 2015; Tedesco et al. 2016; Sannino et al. 2017; Danilovich et al. 2018).

Com relação às actinobacterias, Bruntner et al. (2005) descreveram a nova linhagem de *Streptomyces griseus* NTK 97, que foi isolada a partir de amostra terrestre da Baía Terra Nova, em Edmundson Point na Antártica. Essa bactéria produz o composto antibiótico da classe das "angluciclinonas" denominado "frigociclinona". Ele apresentou uma boa inibição contra bactérias Gram-positivas *Bacillus subtilis* DSM 10 e *Staphylococcus aureus* DSM 20231. Outras actinobacterias isoladas da Antártica com atividades antimicrobianas também foram relatadas por diversos outros autores (O'Brien et al. 2004; Lo Giudice et al. 2007; Rojas et al. 2009; Gesheva and Vasileva-Tonkova 2012; Cheah et al. 2015; Tomova et al. 2015; Lavin et al. 2016; Tedesco et al. 2016).

Já com relação ao filo Firmicutes, Shekh et al. (2011) descreveram a atividade antifúngica de sete isolados bacterianos das regiões ártica e antártica. Estas bactérias exibiram atividade antifúngica contra o patógeno *Candida albicans* cepa NCIM 3471, que costuma apresentar multi-resistência a drogas. Outros autores também encontraram atividade antimicrobiana em isolados do filo Firmicutes na Antártica (O'Brien et al. 2004; Vollú et al. 2014; Guo et al. 2015; Tomova et al. 2015)

Representantes dos filos Bacteroidetes (Mojib et al. 2010; Wong et al. 2011) e Cianobacteria (Taton et al. 2006; Asthana et al. 2009) foram também identificados com a habilidade de produção de compostos antimicrobianos na Antártica.

A maior parte das pesquisas de prospecção de novos compostos se restringe às zonas tropicais e subtropicais e só recentemente estas foram expandidas para as regiões frias (Newman e Hill 2006; Cragg e Newman 2013). A pesquisa de antimicrobianos no ambiente antártico começou no final do século XX e, relativamente, poucos grupos científicos exploram esse nicho. Desde então, apenas algumas moléculas antibióticas foram purificadas e elucidadas. Nenhuma ainda chegou a entrar no mercado; no entanto, a maioria daquelas identificadas corresponde a novas estruturas moleculares. Como resultado, mais trabalho é necessário para a identificação de novas linhagens microbianas, e purificação e caracterização das estruturas químicas desses novos antibióticos, os quais têm grande potencial para aplicações farmacológicas ou industriais (Nuñez-Monteiro et al. 2018).

Pigmentos

Os pigmentos são compostos químicos que refletem a luz na faixa de comprimento de onda da região visível. A cor produzida é devida a estruturas específicas da molécula denominadas cromóforos, isto é, um sistema com alta deslocalização de elétrons que é responsável por absorver certos comprimentos de onda de luz na região visível e fazer com que o composto pareça colorido. As deslocalizações, transições de elétrons $\pi \rightarrow \pi^*$, em produtos naturais, podem envolver vários tipos de grupos, por exemplo: ligações duplas entre carbono-carbono, anéis de benzeno, ligações duplas de carbono-oxigênio, pares isolados de nitrogênio ou oxigênio - e assim por diante (Hari et al. 1994). Os elétrons excitados, das estruturas

que capturaram a energia luminosa, saltam de um orbital externo para um orbital superior; e então, a energia que não é absorvida pela molécula é refletida e / ou refratada para ser capturada pelo olho, que é convertido em impulsos neurais e interpretado como cor pelo cérebro (Delgado-vargas 2000).

Espectro eletromagnético

Entende-se por ultravioleta (UV) a região do espectro eletromagnético emitido pelo sol compreendido entre os comprimentos de onda de 200 a 400 nm. Esta região está conceitualmente dividida em três faixas, o UVC: de 200 a 290 nm; o UVB: de 290 a 320 nm; e o UVA: de 320 a 400 nm (Figura 1).

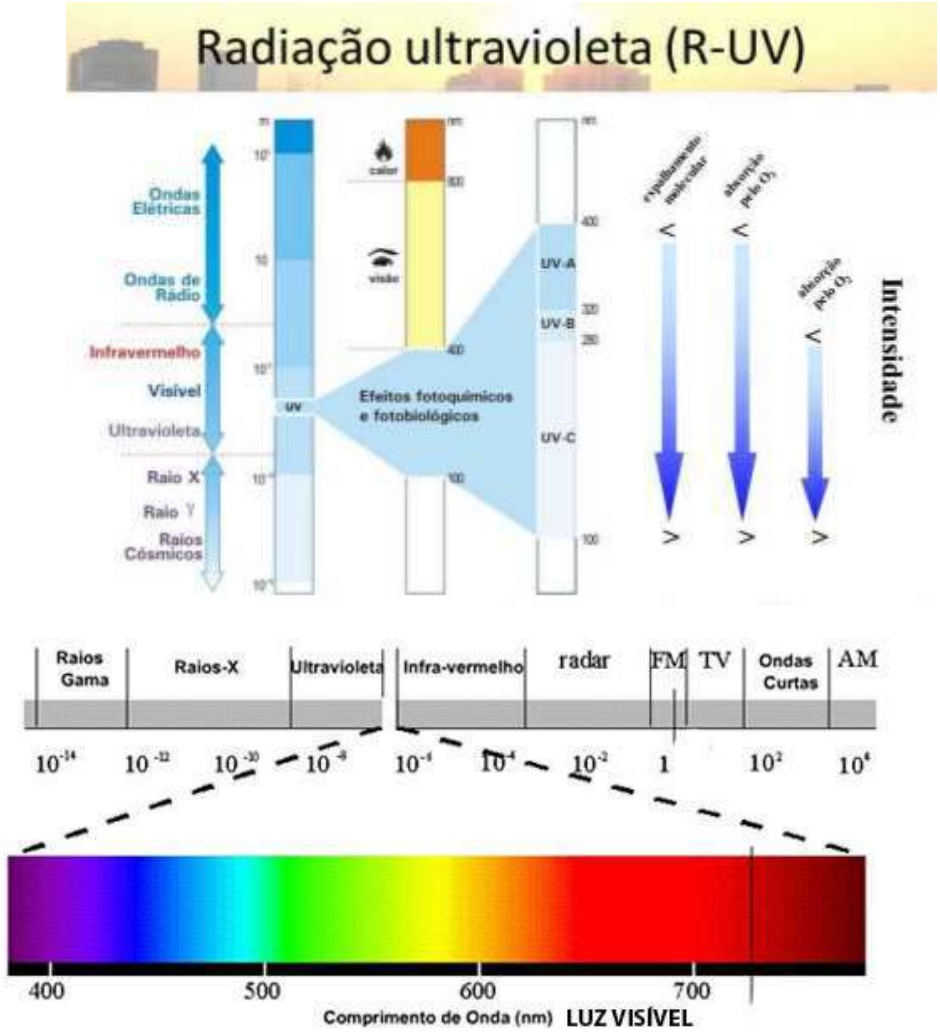


Fig 1. Espectro eletromagnético emitido pelo sol. Faixa de comprimento da luz UV e da luz visível (Fonte: www.solamigo.org/que-e-radiacao-uv Acesso em: 04 dez 2018).

Componentes de origem natural podem apresentar estabilidade limitada e geralmente há pouca informação sobre a segurança de uso. Ao mesmo tempo em que elas podem se comportar como antioxidantes e/ou ter atividade como filtro biológico, elas podem também apresentar atividade pró-oxidante e risco à saúde do consumidor (Cambon et al. 2001; Gaspar e Maia Campos 2006; Spagolla e Tavares 2016). Neste sentido, há necessidade de se estudá-las quanto à segurança, além da sua eficácia.

Fotoestabilidade

Uma molécula possui a habilidade de absorver luz devido a uma transição eletrônica, quando o elétron vai do orbital mais elevado (HOMO) ao mais baixo (LUMO). Os elétrons de alta energia saem do estado fundamental (S_o), nas posições σ, π ou n (pares de elétrons não ligantes) e vão para o estado excitado de mais baixa energia (S_1), correspondente aos orbitais antiligantes σ^*, π^*, devido à absorção de um comprimento de onda específico, migrando de HOMO a LUMO (Nguyen et al. 2013).

Uma molécula, ao absorver um comprimento de onda específico, pode liberá-la por diversos mecanismos. Idealmente ela retorna inalterada ao estado fundamental pela liberação do excesso de energia no relaxamento vibracional não radiativo (calor), ou por processos radioativos (fluorescência ou fosforescência), conseguindo assim fotoestabilidade. A energia também pode ser dissipada por transferência para outras moléculas via fotorreações causando fotossensibilidade. Neste caso, modificações estruturais podem ocorrer, as quais podem ser reversíveis (isomerização *cis*/*trans* ou tautomerização ceto-enólica) ou por efeitos mesoméricos (Nguyen et al. 2013).

Sendo assim, diante de uma triagem de novos candidatos à fotoproteção de produtos naturais, o estudo de fotoestabilidade é imprescindível. Este é realizado com o objetivo de se avaliar os possíveis efeitos da absorção do UV, que podem ser nocivos à pele pela geração de fotorreações quando aplicados topicamente. Um grande indício da não fototoxicidade de uma substância é a manutenção da estrutura molecular após a irradiação solar, ou seja, a fotoestabilidade.

Fototoxicidade

O teste de fototoxicidade por captação do vermelho neutro em fibroblastos murinos 3T3, de acordo com o protocolo OECD TG 432, que apresenta alta sensibilidade e especificidade, é o principal teste validado e normalmente o único ensaio exigido para a determinação da fototoxicidade aguda quando a substância não apresenta potencial fototóxico (Liebsch et al. 2005).

Os intermediários reativos de substâncias fotoinstáveis podem entrar em contato direto com a pele, onde podem se comportar como foto-oxidantes ou promover reações fototóxicas ou foto-alérgicas, como a dermatite de contato. A interação dos produtos de fotodegradação com excipientes ou componentes da pele pode levar à formação de novas moléculas com propriedades toxicológicas desconhecidas. Consequentemente, há uma preocupação crescente sobre a fototoxicidade e fotossensibilidade dos filtros UV (Gaspar et al. 2012).

A fototoxicidade é definida como uma resposta tóxica de uma substância aplicada ao corpo que é induzida ou aumentada após a exposição à luz, ou que é induzida por irradiação da pele após a administração sistêmica de uma substância (OECD 2004).

Historicamente, o potencial para causar fototoxicidade a partir de substâncias aplicadas topicamente era avaliado utilizando modelo animal. Contudo, em 1997 o teste de fototoxicidade 3T3 por captação do Vermelho Neutro (3T3 NRU PT) foi validado pela “European Centre for the Validation of Alternative Methods” ECVAM como adequado para se avaliar o potencial fototóxico de produtos químicos que absorvem no UV/VIS. Este é normalmente o único teste necessário de fototoxicidade quando a substância não é considerada fototóxica. No entanto, são propostos modelos de pele humana reconstituída, por serem semelhantes à barreira da epiderme humana, como uma ferramenta adicional para verificação dos resultados positivos do 3T3 NRU PT, no que diz respeito à mimetização da biodisponibilidade na pele humana, e/ou para o teste de substâncias incompatíveis com o 3T3 NRU PT (Kejlová et al. 2007; Liebsch et al. 2011).

Pigmentos em bactérias da Antártica

A alta freqüência de produção de pigmentos em micro-organismos recuperados de amostras de gelo (Bowman et al. 1997; Rodrigues e Tiedje 2008), águas superficiais marinhas (Agogué et al. 2005; Stafsnes et al. 2010) ou áreas glaciais remotas (González-Toril et al. 2008), indica a importância da pigmentação no processo de adaptação aos ambientes frios e de UV elevado.

Alguns pigmentos são protetores solares extracelulares, outros têm propriedades para dissipar o excesso de energia do UV-B, que alternativamente gerariam singletos de oxigênio nocivos, alguns absorvem UV-B dentro da célula antes que moléculas metabolicamente importantes possam ser danificadas (Wynn-Williams et al. 2001). Pigmentos naturais como os carotenóides já foram identificados com função fotoprotetora (Jagannadham et al. 1996; Jagannadham et al. 2000). Scherer et al. (1988) e Ehling-Schulz et al. (1997) identificaram aminoácidos do tipo micosporina (MAAs) a partir de bactérias capazes de absorver comprimentos de onda entre 310 nm e 360 nm, que podem, portanto, conferir potencial fotoprotetor contra radiação UV (UV-R), bem como radiação visível. Citonemina é outro pigmento encontrado em cianobactérias que confere proteção contra UV-A a 370 nm (Taylor et al. 2010). Como uma alternativa aos pigmentos sintéticos, os pigmentos bacterianos são promissores devido à sua maior biodegradabilidade e compatibilidade com o ambiente, e ainda oferecem caminhos promissores para várias outras aplicações (Venil et al. 2013).

Segundo Hari et al. (1994), os pigmentos naturais encontrados nos sistemas biológicos podem ser divididos em seis grupos: I- carotenoides, II- tetrapirrois, III- biocromos indólicos, IV- biocromos heterocíclicos (outros que não tetrapirrois), V- biocromos heterocíclicos de oxigênio, e VI- quinonas (Figuras 2 a 7). Esses pigmentos mais comuns, identificados entre as bactérias **antárticas,** são discutidos abaixo.

I – Carotenoides

Os pigmentos carotenoides são provavelmente os mais abundantes nas bactérias e pertencem à classe dos *polienos*. Os carotenoides são divididos em dois grupos, carotenos, contendo somente carbono e hidrogênio, e as xantofilas, que contêm também oxigênio (Figura 2). Quase todos os carotenoides são derivados dos tetraterpenos, com ligações duplas conjugadas, que formam o cromóforo, responsável pelas características espectrais da molécula. A biossíntese de seu esqueleto de carbono é baseada na condensação de unidades de isoprenil (Figura 2), conferindo uma variedade de estruturas através de modificações dessa cadeia (Schwender et al. 1996). Eles são produzidos por uma ampla variedade de organismos, desde procariontes não fotossintéticos até plantas superiores, com mais de 750 estruturas diferentes identificadas até o momento (Britton et al. 2004). São encontrados nas cores amarela, laranja ou vermelho e ainda dois incolores, o fitoeno e o fitoflueno. A maioria dos carotenoides tem um esqueleto C40, mas C30 e C50 já foram descritos, especialmente em carotenoides de origem bacteriana (Britton et al. 2004).

Os carotenoides desempenham um papel importante em micro-organismos fotossintéticos, absorvendo a luz e transferindo a energia para a clorofila (Yasushi 1991). A fotoproteção é obtida evitando danos foto-oxidativos às células, interferindo na sequência de eventos, reagindo preferencialmente com o oxigênio singleto oxidante (Goodwin 1988). Além disso, podem modular a fluidez da membrana e proteger do estresse causado pelo frio em bactérias psicrofílicas, mantendo a homeoviscosidade durante flutuações de temperatura (Subczynski et al. 1992; De Maayer et al. 2014). Estudos mostram que a síntese desses pigmentos aumenta em bactérias, *Micrococcus roseus*, cultivadas a 5 °C em comparação com aquelas cultivadas a 25 °C (Chattopadhyay et al. 1997).

Carotenoides apresentam muitas propriedades que os tornam comercialmente atrativos, tais como: alta potência tintorial, não toxicidade, possibilidade de utilização tanto em formulações aquosas quanto lipossolúveis, relativamente estáveis sob condições redutoras, estabilidade na condição ácida e neutra em produtos alimentícios, possibilidade de uso em combinação com outras cores e alto potencial antioxidante. A ação antioxidante dos carotenoides visa combater principalmente as espécies reativas de oxigênio (ROS), fazendo a desativação de radicais singletos de

oxigênio. Os ROS moléculas geradas como um produto natural do funcionamento celular e do metabolismo do oxigênio e têm papéis importantes na sinalização celular e homeostase intercelular. Os efeitos das ROS sobre as células incluem não apenas papéis na apoptose (morte celular programada), mas também efeitos positivos, como a indução de genes de defesa do hospedeiro (Rada and Leto 2008). No entanto, os níveis de ROS podem aumentar dramaticamente devido ao estresse ambiental, como radiação ionizante, UV ou exposição ao calor (Devasagayam et al. 2004). Este aumento pode resultar em dano celular significativo, que cumulativamente constituem o estresse oxidativo. ROS podem danificar DNA, RNA e proteínas, contribuindo assim para a fisiologia do envelhecimento.

Assim, os carotenoides são utilizados como corantes alimentares, suplementos alimentares e para fins cosméticos e farmacêuticos (Hari et al. 1994; Britton 1995; Mercadante 2008; Houghton e Hendry 2012). A astaxantina é um exemplo de carotenoide que tem valor comercial como suplemento alimentar para humanos e como aditivo alimentar usado para a ração de salmonídeos.

Diferentes tipos de carotenoides foram identificados a partir de bactérias isoladas da Antártica, sendo alguns tratados abaixo.

Pigmentos de bactérias do gênero *Pedobacter* isoladas de amostras genéricas da Antártica foram identificados como pirroxantina, fucoxantina, violaxantina e 3-sulfato-nostoxantina. O extrato da mistura dos pigmentos possuía uma forte capacidade antioxidante, verificada pela taxa de consumo de oxigênio, e protegia a bactéria contra danos oxidativos causados por altos níveis de radiação UVB (Correa-Llantén et al. 2012).

Bactérias pigmentadas do grupo Flexibacter-Bacteroides-Cytophaga foram também isoladas de amostras da Antártica. Membros desses filos são particularmente reportados por conterem pigmentos do tipo carotenoides, como por exemplo, a família Flavobacteriaceae (Bowman et al. 1997; Humphry et al. 2001; Dieser et al. 2010; Mojib et al. 2010). Outras bactérias do filo Bacteroidetes, identificadas como *Sphingobacterium antarcticus* e *Sphingobacterium multivorum*, com pigmentação amarelo/laranja, foram isoladas de amostras de solo e produziram zeaxantina, β-criptoxantina e β-caroteno (Jagannadham et al. 2000). A linhagem *Gillisia limnaea* DSM 15749 de cor amarela, isolada a partir de tapetes microbianos no Lago Fryxell,

Antártica, apresentaram os carotenoides 3" -hidroxi-2'-isopentenil-saproxantina bem como zeaxantina (Takatani et al. 2015).

Outros carotenoides pouco conhecidos vêm sendo descobertos nas bactérias antárticas. Por exemplo, o gênero *Hymenobacter* produz carotenoides raros, como a plectaniaxantina, saproxantina e as variações de carotenoides que compartilham um esqueleto comum de 2'-hidroxiflexixantina (Klassen e Foght 2008; Klassen e Foght 2011).

A xantorodopsina é um complexo de proteína / carotenoide (salinixantina) que atua na transferência de energia de carotenoides coletores de luz para clorofilas, funcionando como um pigmento antena baseado em bombas de íons de retina e fotorreceptores (Balashov e Lanyi 2007). O gene para o grupo xantorodopsina foi relatado na espécie *Octadecabacter antarcticus* e foi expresso em *E. coli* C43 (Imasheva et al. 2009; Vollmers et al. 2013).

A bactéria púrpura, *Rhodoferax antarcticus* Fryx1, isolada de coluna de água sob o gelo do Lago Fryxell - Antártica, produz os carotenoides esferoideno, esferoidenona, metoxi-esferoidenona e espiriloxantina (Jung et al. 2004).

Além disso, uma série de carotenoides não identificados é descrita em diferentes bactérias. Leiva et al. 2015 isolaram as bactérias amarelas *Kocuria palustris* AUE4, e *Citricoccus zhacaiensis* ICE1 e ICB8 de macroalgas marinhas na Antártica. Os pigmentos produzidos por estas bactérias foram identificados como carotenoides e os mesmos isolados apresentaram atividade antimicrobiana contra *Zobellia laminarie*. Outras bactérias pigmentadas do mesmo estudo, como *Brachybacterium rhamnosum* MHE3, apresentaram atividade antimicrobiana contra *Citricoccus zhacaiensis*; e a bactéria *Agrococcus baldri* MHJ5 apresentou atividade antimicrobiana contra *Kocuria palustris*. Bowman e Nichols 2002 isolaram a bactéria alaranjanda *Aequorivita*, um membro da família Flavobacteriaceae, de habitats antárticos terrestres e marinhos. Outros estudos reportaram o isolamento das bactérias alaranjadas *Kocuria polaris* (Reddy et al. 2003a) *Antarcticimonas flava* IMCC3175 (Yang et al. 2009) *Sejongia marina* (Lee et al. 2007) de amostras de água do mar da Antártica, entre outras (Vincent et al. 1993; Peeters et al. 2011).

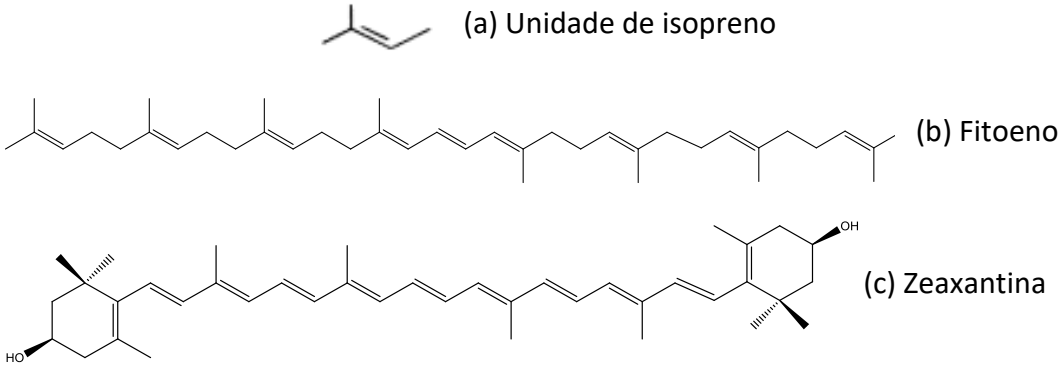


Fig 2. Estrutura molecular da subunidade de isopreno (a), exemplos de caroteno - fitoeno (b) e xantofila - zeaxantina (c).

II- Tetrapirróis

Os tetrapirrois são compostos orgânicos formados por quatro anéis pirrólicos, ou seja, é um composto heterocíclico aromático insaturado com quatro anéis de carbono. Os pigmentos tetrapirrois estão associados principalmente às bacterioclorofilas A e B de bactérias fotossintetizantes (Labrenz et al. 1999; Labrenz et al. 2005; Yu et al. 2011). Outros pigmentos acessórios para a fotossíntese, como as ficobilinas, atuam no metabolismo energético como o principal meio de captação de luz em cianobactérias (Madigan et al. 2012). Alguns lagos na Antártica são dominados por bacterioplânctons, que têm como representantes as sulfobactérias fotossintetizantes *Chlorobium vibrioforme* e *Chlorobium limnicola*. O mundo fotossintético das cianobactérias incluem as espécies dos gêneros *Phormidium*, *Oscillatoria*, *Cylindrospermum* e *Nostoc* (Volkman et al. 1988; Cowan and Tow 2004). A bacterioclorofila também está presente em bactérias fototróficas púrpuras que desempenham um papel importante na produtividade primária de lagos permanentemente congelados nos vales secos antárticos (Karr et al. 2003).

Os tetrapirrois cíclicos também são encontrados em pigmentos do grupo heme como hemoglobina (Figura 3), leg-hemoglobina, mioglobina e citocromos. O pigmento vermelho citocromo C3 foi isolado da bactéria antártica *Shewanella frigidimarina* e está associado à adaptação térmica e / ou à radiação. Além disso, pigmentos como o citocromo C3 permitem que essa espécie bacteriana use um metabolismo anaeróbico e férrico (Martín-Cerezo et al. 2015).

Os mono, di e tripirrois são raros, embora o pigmento bacteriano vermelho prodigiosina de *Serratia marcescens* seja um tripirrol linear (Figura 3). Representantes desta espécie foram detectados no solo de Schirmacher Oasis na Antártica através de bibliotecas do gene RNAr 16S (Shivaji et al. 2004). A família das prodigioninas possui uma ampla variedade de propriedades biológicas, incluindo atividades antibacterianas, antimaláricas, antifúngicas, imunossupressoras e anticancerígenas (Bennett and Bentley 2000; Montaner and Pérez-Tomás 2003; Suryawanshi et al. 2015).

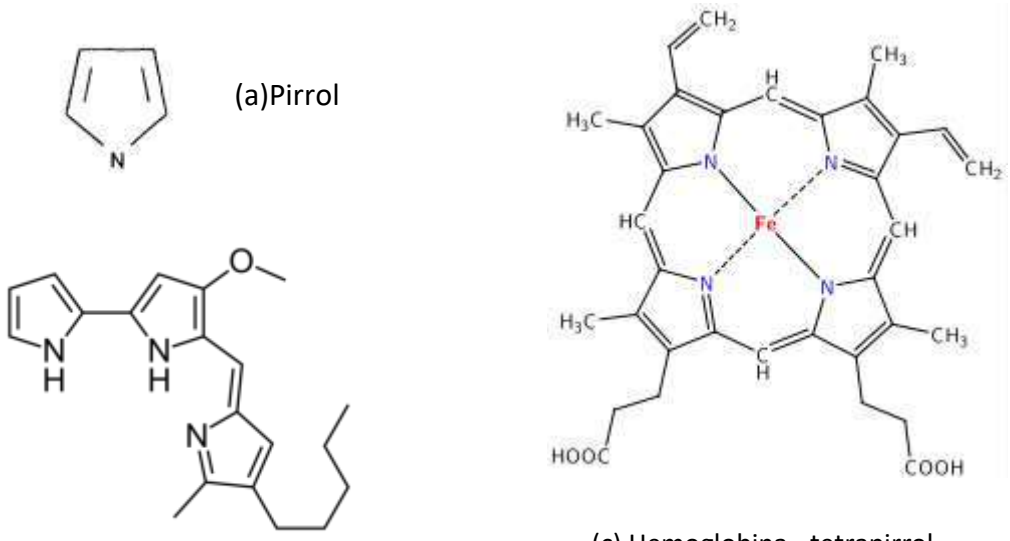


Fig 3. Estrutura da subunidade pirrol (a); e exemplo de tripirrol, a prodigiosina (b) e tetrapirrol cíclico, a hemoglobina (c).

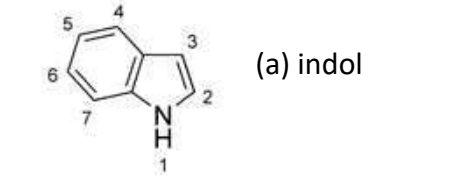
III- Biocromos Indólicos

Os biocromos indólicos contêm um núcleo benzo-pirrol condensado. As melaninas são os seus representates (Figura 4). Estes compostos poliméricos nitrogenados são sintetizados por diferentes organismos. Vários tipos de melaninas são descritos: eumelaninas (preto ou marrom), feomelaninas (amarelo-vermelho), alomelaninas e piromelaninas (Nicolaus et al. 1964; Banerjee et al. 2014).

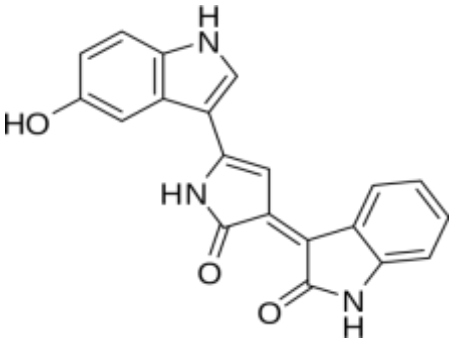
A produção de melanina é uma característica comum entre os membros do gênero *Lysobacter*. O pigmento de *L. oligotrophicus* isolado da Antártica foi identificado como melanina solúvel em água (Lo-melanina); este pigmento diluído no meio de cultura aumentou a taxa de sobrevivência de *Escherichia coli* sob condição de

irradiação UV (Kimura et al. 2015). Outro exemplo é a actinobacteria *Nocardiopsis antarcticus*, que sintetiza alta concentração de melanina intracelular (Abyzov et al., 1983).

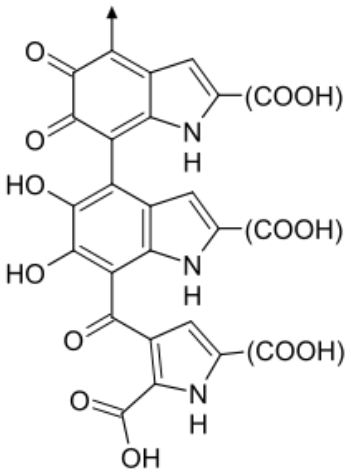
O pigmento violaceína é um derivado indol (Figura 4) que apresenta a coloração violeta. Já foi isolado de bactérias *Janthinobacterium lividum* que habitam áreas terrestres das ilhas Antárticas (Wynn-Williams 1983; Kämpfer et al. 2008). O pigmento roxo é amplamente descrito com propriedades multifuncionais como atividades antitumorais, antiparasitárias, antiprotozoárias, antivirais, antibacterianas e também antioxidantes (Matz et al. 2004; Duran et al. 2007). O mecanismo antitumoral ainda está sendo estudado e pode ativar a apoptose nas linhagens de célula de leucemia HL60 (Nakamura et al. 2002).



(a) indol



(c) Violaceína



(b) Melanina

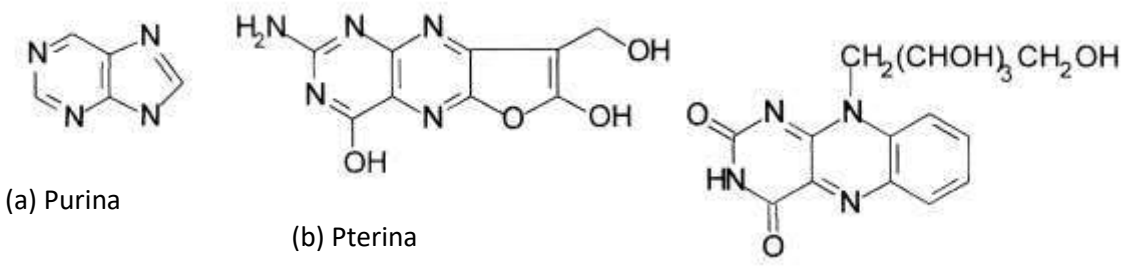
Fig 4. Estrutura da subunidade indólica (a) e exemplo de biocromos indólicos, a melanina (b) e a violaceína (c).

IV- Biocromos Heterocíclicos (exceto tetrapirrois)

O grupo de biocromos heterocíclicos é representado por compostos com estruturas muito complexas encontradas em fenazinas, purinas, pterinas, flavinas, fenoxazinas e betalaínas (Figura 5) (Delgado-Vargas et al. 2000).

As fenazinas são compostos aromáticos contendo nitrogênio tricíclico que variam de amarelo, laranja, vermelho a até marrom. Mais de 100 tipos de fenazinas naturais e biologicamente ativas, sintetizadas principalmente por espécies de *Streptomyces* e *Pseudomonas*, são conhecidas (Gerber 1969; Dakhama et al. 1993; Schneemann et al. 2011). Um isolado de *Pseudomonas aeruginosa* associado à esponja coletado no mar antártico produziu dois tipos de fenazinas. Estes pigmentos exibiram propriedades antibacterianas contra *Bacillus cereus* (MIC por ensaio em disco <0,5 µg / mL) (Jayatilake et al. 1996).

O pigmento piocianina possui coloração azul ou verde, com consideráveis propriedades tóxicas em uma ampla gama de organismos alvos, compreendendo bactérias, leveduras, insetos, nematoides e plantas. Já foi observado principalmente nos gêneros *Pseudomonas* e *Streptomyces* (Malpartida and Hopwood 1984). A piocianina foi detectada em uma bactéria Gram-negativa isolada da Antártica, com morfologia incomum, próxima filogeneticamente de *Flavobacterium* (McMeekin 1988).



(a) Purina

(b) Pterina

(c) Riboflavina

Fig 5. Estrutura dos biocromos heterocíclicos que não tetrapirrois, exemplos: purina (a), pterina (b) e riboflavina (c).

V- Biocromos Heterocíclicos Oxigênicos (Derivados do Benzopirano) - Antocianinas e Flavonoides

Os flavonoides e antocianinas (Figura 6) são pigmentos encontrados principalmente em plantas. Não há relatos de bactérias, que não sejam biotecnologicamente transformadas, que produzam esses tipos de compostos.

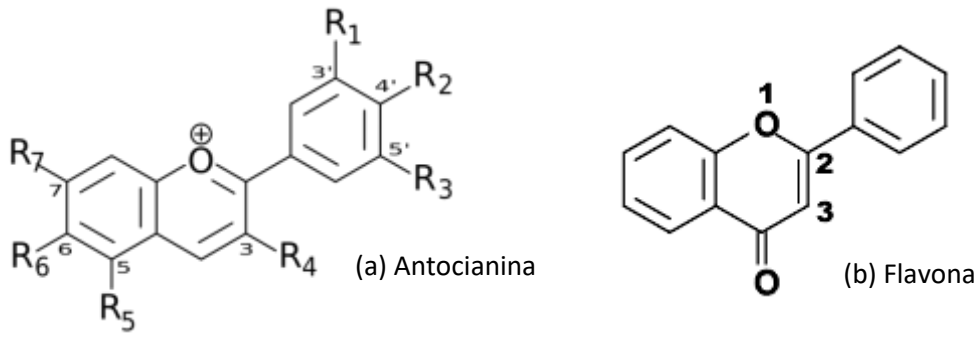


Fig 6. Estrutura de biocromos heterocíclicos oxigênicos. Exemplos: antocianina (a) e flavona (b).

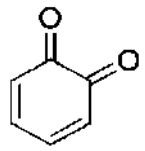
VI- Quinonas

A estrutura básica das quinonas é a de uma dicetona cíclica insaturada derivada de compostos aromáticos com qualquer rearranjo necessário de ligações duplas (Figura 7). Exemplos incluem antraquinonas, benzoquinonas e naftaquinonas.

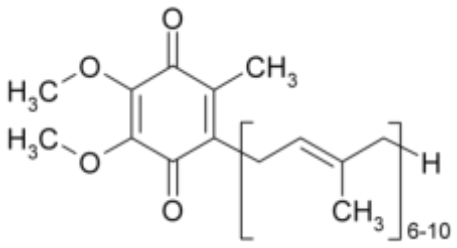
Antraquinonas constituem o maior grupo de quinonas na natureza. A antraquinona mais comumente encontrada é a emodina, encontrada em fungos superiores, líquens, angiospermas e insetos. As antraciclinonas são antraquinonas modificadas, por exemplo, aclavinona encontrada em Streptomycetos (Hari et al. 1994). As duas benzoquinonas mais importantes são a plastoquinona, que ocorre nos cloroplastos de plantas superiores e algas, e a ubiquinona, que tem ocorrência quase universal em organismos vivos. As naftaquinonas naturais incluem a filoquinona (vitamina K), encontrada nas plantas superiores, e a menaquinona (vitamina K2), que ocorre nas bactérias. Em geral, as quinonas produzem colorações amarelas, marrons ou vermelhas, mas os sais de quinona apresentam cores azul, púrpura ou verde (Hari et al. 1994; Delgado-Vargas et al. 2000). As quinonas têm aplicações industriais como corantes (hena, alizarina, alcanina, shikonina), corantes alimentares (ácido carmínico)

e produtos medicinais (naftaquinonas e antraquinonas são usados como purgantes, e aclavinona mostra atividade antibiótica) (Hari et al. 1994).

Na Antártica foi detectada a produção de quinonas em espécies de *Sphingomonas* isoladas de ar e poeira. Esta bactéria apresentou pigmentação laranja que consistia predominantemente de ubiquinona Q-10 (Busse et al. 2003). *Shewanella frigidimarina* e *Shewanella livingstonensis* isoladas de áreas costeiras antárticas continham ubiquinonas, menaquinonas e metilmenaquinonas (Bozal et al. 2002). Ubiquinona e menaquinona (MK) servem como coenzimas respiratórias nos sistemas de transporte de elétrons de organismos vivos. A MK é a quinona mais amplamente dispersa na árvore filogenética, em particular, em arqueias e bactérias. Ela parece assim representar o tipo ancestral de quinonas nos sistemas bioenergéticos (Schoepp-Cothenet et al. 2009). *Leifsonia rubra* e *Leifsonia aurea*, isoladas de tapetes de cianobactérias na Antártica, contêm MK-11 (Reddy et al. 2003b), *Hymenobacter roseosalivarius*, isolado de solos antárticos continentais e arenito, produz MK-7 (Hirsch et al. 1998) e *Kocuria polaris* CMS 76or, uma bactéria pigmentada laranja isolada de tapetes de cianobactéria na Antártica, apresentou o MK-7, MK-8 e pequenas quantidades de MK-6 e MK-9 (Reddy et al. 2003a).



(a) Benzoquinona



(b) Ubiquinona Q10

Fig 7. Exemplos de estrutura de quinonas: benzoquinona (a) e ubiquinona Q10 (b).

Outros pigmentos:

Citonemina (do inglês "Scytonemin")

Citonemina é um pequeno alcaloide lipossolúvel (Figura 8a) de cor amarelo-amarronzada a vermelho escuro presente na matriz extracelular de várias cianobactérias. Foi identificada pela primeira vez em *Nostoc commune,* isolada da

periferia de uma lagoa de água doce na Ilha Bratina, McMurdo Ice Shelf (Garcia-Pichel et al. 1992). Muitas outras cianobactérias expostas a altas doses de insolação também apresentaram produção de citonemina (Ehling-Schulz et al. 1997; Taylor et al. 2010; Vincent et al. 2016). O pigmento está associado a mecanismos de proteção contra radiação ultravioleta (UV) de onda curta, a qual absorve 88% da UV-A em 370 nm *in vivo*, minimizando a produção de espécies reativas de oxigênio e prevenindo lesões de DNA (Rastogi et al. 2015).

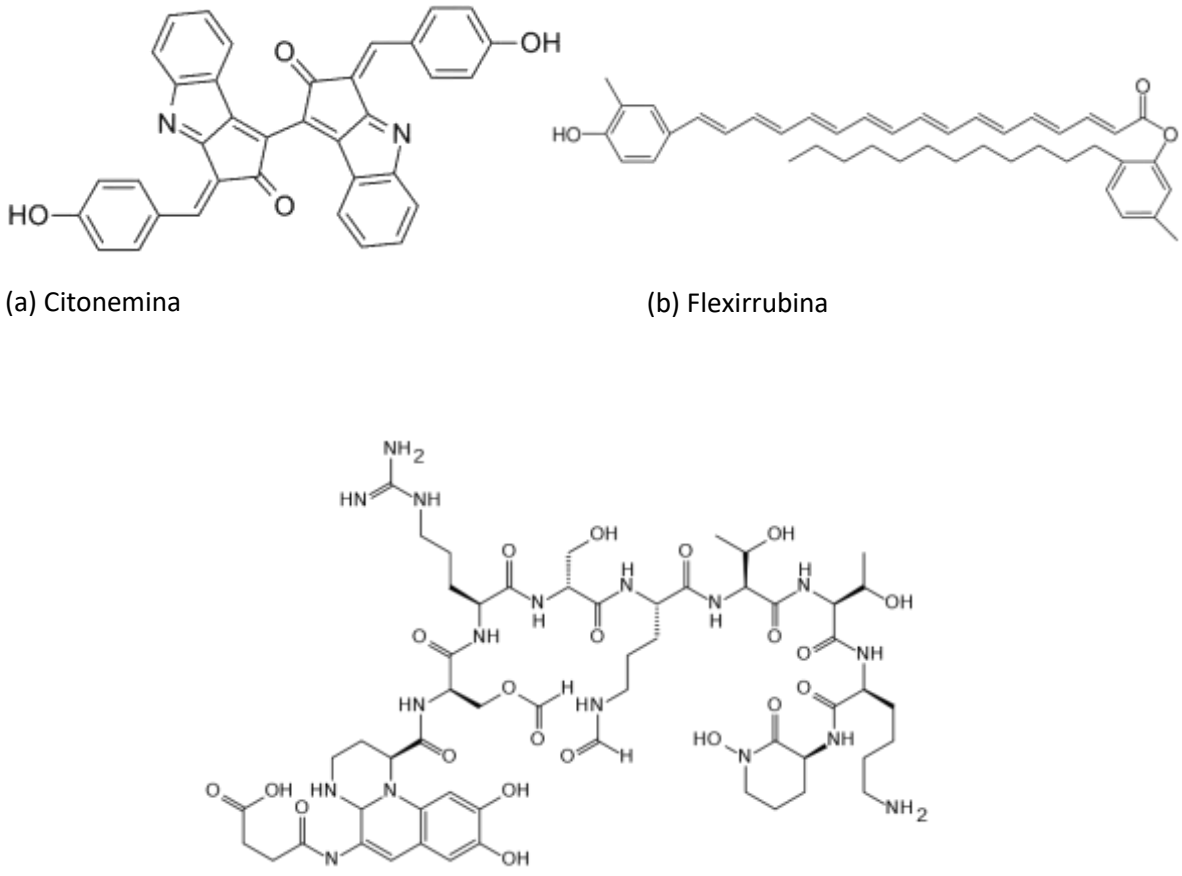
Flexirrubina

Os pigmentos de flexirrubina (Figura 8b) são amarelos a alaranjados e estão presentes em *Flavobacterium* e *Flexibacter* (Bacteroidetes). As flexirrubinas são estruturas relacionadas às dos carotenoides, chamadas aril polienos (APEs) (Schöner et al. 2016). Estes compostos são ácidos carboxílicos polienos com ligação do tipo éster com um sistema dialquilresorcinol (DAR) (Jehlička et al. 2013; Jehlička et al. 2014). A bactéria *Flavobacterium* sp. Ant342 (F-YOP), isolada dos lagos de Schirmacher Oasis, leste da Antártica, produzem um pigmento flexirrubina amarelo-alaranjado. Este pigmento exibiu atividade antibacteriana contra alguns micro-organismos com MICs de 3,6 µg/mL contra *Mycobacterium smegmatis* mc2155 virulento, 2,6 µg/mL contra *Mycobacterium tuberculosis* mc26230 virulento, e 10,8 µg/mL para *Mycobacterium tuberculosis* H37Rv (Mojib et al. 2010).

Pioverdinas

As piodoverdinas (Figura 8c) são pigmentos fluorescentes amarelo-esverdeados, solúveis em água, excretados por espécies de *Pseudomonas*. O cromóforo deste pigmento é um derivado da quinolina, o que confere à molécula cor e fluorescência (Meyer 2000). Devido ao seu aspecto brilhante, torna-se muito fácil de ser detectado no meio King B (King et al. 1954). A pioverdina desempenha uma importante função fisiológica como sideróforo, um poderoso captador e transportador de ferro (III) presente estritamente em bactérias aeróbicas (Meyer 2000; Winkelmann 2002). Linhagens de *Pseudomonas* isoladas de amostras de gelo ricas em algas na Antártica, relacionadas com *Pseudomonas mucidolens* e *Pseudomonas synxantha*

(IC038 e A177), produziram pigmento fluorescente verde semelhante à pioverdina (Bowman et al. 1997).



(a) Citonemina (b) Flexirrubina

(c) Pioverdina

Fig 8. Estrutura molecular de outros pigmentos encontrados em bactérias da Antártica: citonemina (a), flexirrubina (b) e pioverdina (c).

OBJETIVOS

- Levantamento taxonômico da diversidade de bactérias isoladas de diferentes amostras da Antártica.
- Prospecção de metabólitos secundários com atividade antimicrobiana, antitumoral, antiparasitária e fotoprotetora a partir de bactérias da Antártica.
- Análise e identificação de pigmentos com potenciais de aplicação biotecnológica encontrados nessa comunidade.

DESCRIÇÃO DO LOCAL DE COLETA E DAS AMOSTRAS DA ANTÁRTICA

Pontos de coleta e material de isolamento de bactérias

A Antártica é dividida em três grandes regiões: i) a Antártica marítima - compreendida pela faixa oceânica ao longo do continente, ii) a faixa peninsular e insular - compreendida pela península Antártica e arquipélagos no entorno do continente, e iii) a Antártica continental, onde se encontra a porção terrestre 98% coberta por gelo e que possui as condições mais extremas do planeta, onde poucas formas de vida são encontradas (Figura 9).

As amostras coletadas para o desenvolvimento dessa tese foram obtidas nas Ilhas Shetlands, na península Antártica (Figura 10), onde as condições ambientais, apesar de severas, são mais amenas que na porção continental. As coletas das amostras foram realizadas pelo Dr. Alysson Duarte (UFAL) durante a OperAntar XXXII e pelo doutorando Tiago Rodrigues e Silva durante a OperAntar XXXIV, ambos participantes do programa MycoAntar. Exemplos de materiais coletados para isolamento podem ser observados na Figura 11.

Fig 9. Sub-regiões do continente Antártico (Foto: An orthographic projection of NASA's Blue Marble data set).

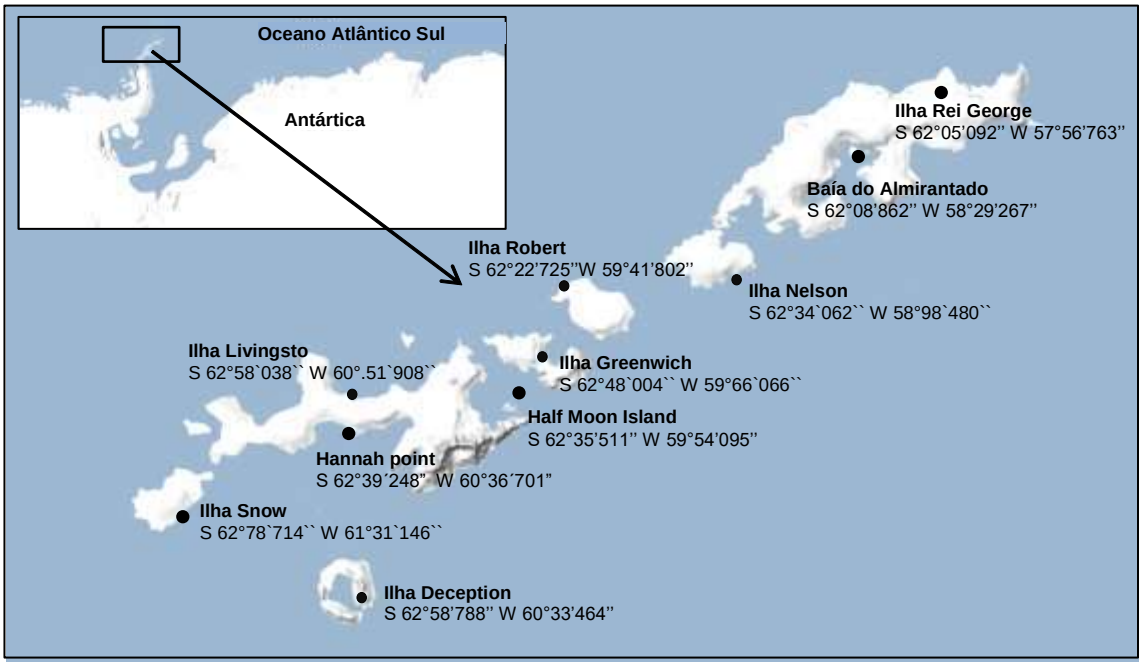


Fig 10. Localização geográfica e pontos de amostragem (•) nas ilhas Shetlands do Sul (Antártica).

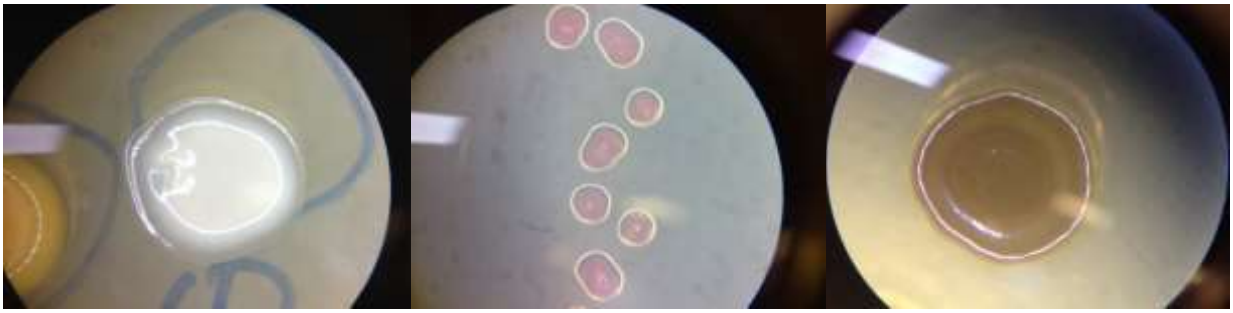
Líquens Invertebrados Solo de pinguineira

Solo associado a raiz Solo Sedimento

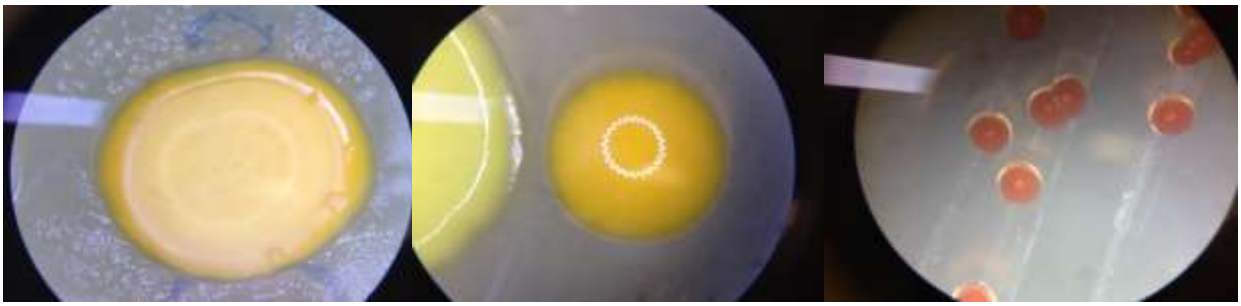
Moluscos Algas Biofilmes

Fig 11. Exemplos de amostras coletadas para isolamento (Foto: Tiago R. Silva 2018).

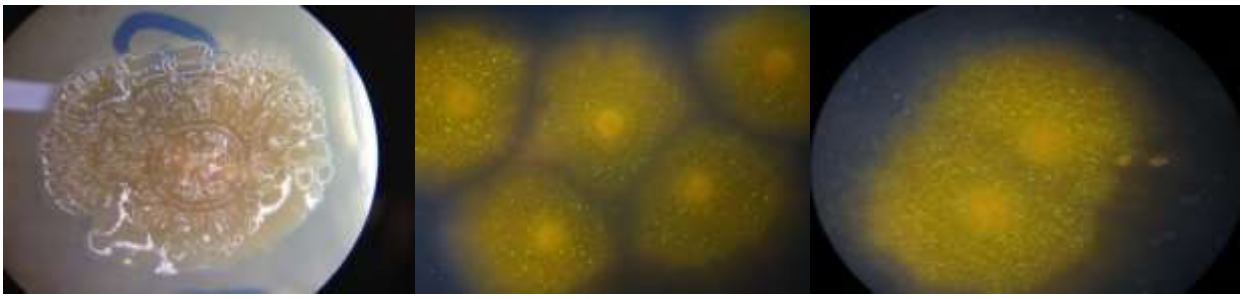
As Figuras 12 e 13 ilustram e exemplificam algumas bactérias pigmentadas isoladas das amostras provenientes da Antártica, que foram estudadas nessa tese.



Arthrobacter livingstoniensis 424 *Arthrobacter agilis 49-CYT* *Arthrobacter alpinus 366*



Rhodococcos luteus 267 *Arthrobacter sp. 255* *Arthrobacter agilis 50Cyt*



Pseudoalteromonas arctica 466 *Celullophaga fucicola 416*

Fig 12. Isolados bacterianos pigmentados da Antártica (Foto: Tiago R. Silva, 2018).

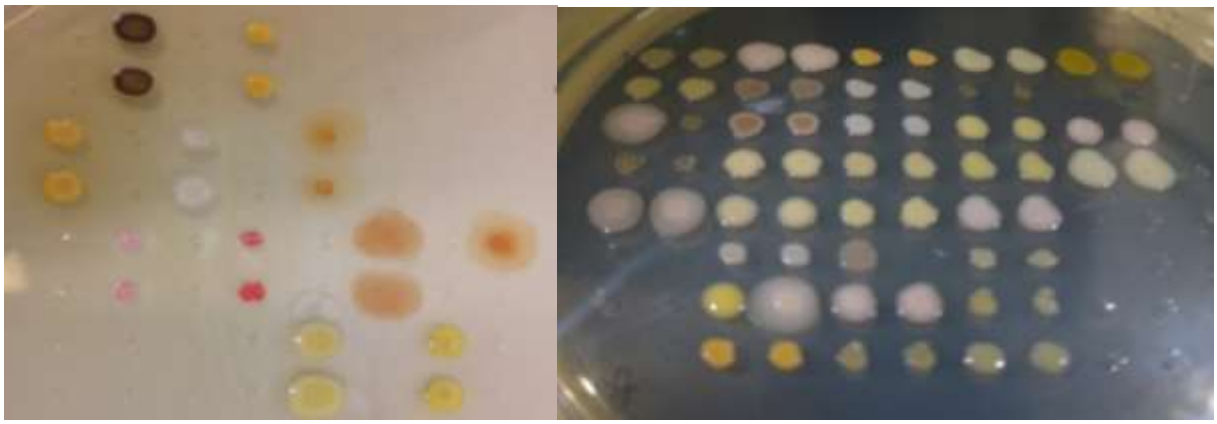


Fig 13. Exemplos dos isolados bacterianos pigmentados da Antártica (Foto: Tiago R. Silva, 2018).

APRESENTAÇÃO DA TESE

Este trabalho é o resultado da busca por novos compostos a partir da microbiota isolada de diferentes amostras do ambiente Antártico.

No Capítulo 1 são apresentados os resultados do levantamento taxonômico dos isolados bacterianos obtidos a partir das diferentes amostras da Antártica. Foi realizada uma triagem por compostos antimicrobianos a partir da microbiota cultivável recuperada e a bactéria identificada como *Pseudomonas* sp. 99 mostrou ser produtora de compostos com atividades antimicrobiana, antiproliferativa e antiparasitária. Este capítulo está na forma de artigo publicado na revista Polar Biology (Silva et al. 2018a).

No **Capítulo 2** são apresentados os resultados obtidos para os pigmentos das bactérias isoladas da Antártica nos testes de resistência a UV. Esses pigmentos foram testados para ação antioxidante, de fotoestabilidade e fotoproteção. Além disso, foi realizada a identificação dos pigmentos, incluindo da fração que se mostrou eficaz na proteção contra UV e não apresentou fotoxicidade em testes *in vitro*. Esse artigo está em fase final de revisão na revista Marine Biotechnology.

No **Capítulo 3** são apresentados os resultados da pigmentação da bactéria iridescente brilhante identificada como *Cellulophaga fucicola* 416. O artigo foi publicado na forma de “Short Communication” na revista Antoine van Leeuwenhoek (Silva et al. 2018b).

Por fim, as principais conclusões obtidas do trabalho serão discutidas de maneira integrada no tópico “Discussão e Perspectivas Futuras”.

CAPÍTULO I

Bacteria from Antarctic environments: diversity and detection of antimicrobial, antiproliferative, and antiparasitic activities

Bactérias do ambiente Antártico: diversidade e detecção de atividade antimicrobiana, antiproliferativa e antiparasitária

Polar Biology
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ORIGINAL PAPER

Bacteria from Antarctic environments: diversity and detection of antimicrobial, antiproliferative, and antiparasitic activities

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Abstract

Microorganisms dominate most of Antarctic ecosystems and play a crucial role in their functioning. They are called extremophilic microorganisms with unique and versatile metabolic properties with possible biotechnological applications in several areas. The aim of the present study was to identify psychrotolerant microorganisms from Antarctic continent samples and to screen them for antimicrobial effects. Phylogenetic analyses revealed that most isolates were closely related to recognized species, including those recovered previously from Antarctica, which belonged to the major phyla Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (classes Alpha, Beta, and Gammaproteobacteria). A total of 326 bacterial isolates, distributed in 39 different genera, were recovered and identified based on sequencing of the 16S rRNA gene. The main representative genera were *Arthrobacter*, *Psychrobacter*, *Pseudoalteromonas*, and *Rhodococcus*. Antimicrobial screening revealed fifteen isolates capable of inhibiting growth of at least one of the indicator strains: *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Candida albicans*. One psychrotolerant bacterium, *Pseudomonas* sp. isolate 99, showed a broad antimicrobial range, in addition to antiproliferative and antiparasitic activity. Overall, the small number of antibiotic-producing isolates obtained and the weakness of their inhibition halos corroborated previous findings suggesting that cold-loving bacteria from Antarctica are not as good as their relatives from mesophilic environments for antimicrobial prospecting. Nonetheless, antiproliferative and antiparasitic results observed are promising and suggest that there is an untapped wealth in Antarctic environments for bioprospecting compounds with pharmaceutical potential application.

Keywords Bioprospecting · Bioactive compounds · Cold environments · 16S rRNA genes · *Pseudomonas*

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00300-018-2300-y) contains supplementary material, which is available to authorized users.

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Introduction

Antarctica is located south of the Antarctic Polar circle and is called the continent of extremes. It has the most extreme weather conditions, including low temperatures which may reach up to − 89 °C, high incidence of UV radiation, and water and nutrient deficit (Margesin et al. 2008; Margesin and Miteva 2011). It has the highest average altitude and is the most isolated of all continents. This geographic isolation probably resulted in the selection/speciation of unusual microorganisms, and also prevented their spread to other continents. That unique biome means that much of its biological heritage cannot be found anywhere else on the planet (Nichols et al. 1999).

Without human colonization, the continent still preserves the records of historical processes by which the earth passed in the last millennia. Thus, there is a possibility of confrontation and comparison, past and present, between environmental conditions, both biological and climatic (Wilmotte et al. 2012). So, an effort to provide a "database" on microbial composition is necessary to forecast possible future changes in diversity and taxonomic composition, as a result of variations in the ecosystem and/or human introductions.

Microorganisms dominate most Antarctic ecosystems and play a crucial role in their functioning and primary productivity. Several studies have shown that there is great biodiversity in the polar regions and different types of biological activities (Graumann et al. 1996; Morita et al. 1999; Deming 2002b; Bowman 2004). Cultivable bacteria from Antarctica have been studied by several authors (Bowman et al. 1997; Van Trappen et al. 2002; Webster and Bourne 2007; Chong et al. 2012; Huang et al. 2013; Shivaji et al. 2013). The main representative bacterial groups isolated belonged to four major phyla: Actinobacteria, Bacteroidetes, Proteobacteria, and Firmicutes. However, compared to the temperate and tropical regions, and despite their ecological importance, little is known about diversity of microbes and their geographical distribution in Antarctic systems (Taton et al. 2003; Vyverman et al. 2010).

Physiological adaptations allowed microorganisms to thrive in Polar Regions. Cold-shock proteins empower specific activities as the temperature drops. Cold active enzyme tends to reduce activation energy leading to high catalytic efficiency (Gerday et al. 1997). Expression of antifreeze-nucleating proteins and exopolysaccharide promotes survival at subzero temperature (Nichols et al. 1999; Muryoi et al. 2004; Nichols et al. 2005). In addition, increase in the proportion of unsaturated fatty acids in the cellular membranes helps to maintain a semifluid state at low temperatures (Deming 2002a; Häggblom and Margesin 2005; Newman and Hill 2006; Margesin et al. 2007, 2008; Shivaji and Prakash 2010).

These particular traits and unique adaptations already revealed suggest that the Antarctic environment represents a huge pool of microbial biodiversity and exploitable biotechnology. This untapped diversity has resulted in increasing interest in the study of cold-adapted microorganisms exploiting their ability to produce novel metabolites/compounds with potential biotechnological applications (Kennedy et al. 2008); for example, antimicrobial compounds have already been identified (Bruntner et al. 2005; Shekh et al. 2011; Liu et al. 2013) as well as antiproliferative molecules (Mojib et al. 2010, 2011), which could be used as anticancer drugs.

The evolution of antibiotic resistance by major human pathogens has made the first antibiotics, and most of their successors, mostly ineffective (Spellberg et al. 2004; 2013). Despite the significant efforts in the search for new antibiotics, bacteria are getting resistant faster than the appearance of new drugs to the market.

The search for new drugs is therefore of great importance to modern society and a promising alternative is to explore biodiversity in environments with unique characteristics, which contain microorganisms with particular physiological and metabolic properties that allow them to adapt and survive in inhospitable conditions, such as the Antarctic continent.

This work aimed to identify a broad collection of psychrotolerant bacteria isolated from diverse Antarctic samples and evaluate their potential for producing antimicrobial, antiproliferative, and antiparasitic compounds.

Materials and methods

Sampling

Samples used in this study were collected during an expedition to Antarctica in the austral summer (2013 and 2015) by the MycoAntar—Brazilian Antarctic Program team. The sources and places from which samples were obtained are detailed in Table 1 and Fig. 1. Samples were collected aseptically and placed in sterile plastic bags. Terrestrial samples as soil, penguin soil, sediment, and biofilm were frozen right after sampling and lichen, sponges, sea star, and other bryozoans were kept refrigerated at 4 °C and transported to the Division of Microbial Resources (DRM), Campinas, Brazil, for further processing. A flowchart depicting the methodological strategy adopted in this work for screening the bioactive compounds is shown in Fig. 2.

Bacterial isolation and identification

Isolation of bacteria was carried out on R2A agar (Yeast extract 0.5 g, Proteose Peptone 0.5 g, Casamino acids 0.5 g, Glucose 0.5 g, Soluble starch 0.5 g, Na-pyruvate 0.3 g,

Table 1 Data related to marine and terrestrial Antarctic samples

Sample	Site	GPS data
Marine sediment		
Deception sediment	Deception Island	S 62°58′788″ W 60°33′464″
Martel Bay sediment	Martel Bay, King George Island	S 62°08′862″ W 58°29′267″
Punta Hannah sediment	Punta Hannah Livingston Island	S 62°39′149″ W 60°38′230″
Rei George sediment	King George Island	S 62°05′948″ W 58°23′622″
Biofilm		
Soil—biofilm 3	Half Moon Island	S 62°35′511″ W 59°54′095″
Whalers Bay soil biofilm	Whalers bay (WB) Deception Island	S 62°58′788″ W 60°33′464″
Penguin´s Nest	Barrientos	S 62°24′392″ W 59°44′404″
Soil		
Waterlogged soil (1) 1.6 °C	King George Island—Punta Turret	S 62°05′092″ W 57°56′763″
Waterlogged soil (2) 4 °C	King George Island—Punta Turret	S 62°05′092″ W 57°56′763″
Sponge		
Sponge	Punta Hannah—Livingston Island	S 62°39′149″ W 60°38′230″
Sponge1	Punta Hannah—Livingston Island	S 62°39′149″W 60°38′230″
Sponge2	Punta Hannah—Livingston Island	S 62°39′149″ W 60°38′230″
Sea Star	Half Moon Island	S 62°35′511″ W 59°54′095″
Nacella concinna	Robert Island	S 62°22′725″ W 59°41′802″
Marine invertebrate		
Invertebrate BRHM	Half Moon Island	S 62°35′511″ W 59°54′095″
Invertebrate 16	Punta Hannah—Livingston island	S 62°39′149″ W 60°38′230″
Invertebrate 14	Half Moon Island	S 62°35′511″ W 59°54′095″
Lichen and seaweed		
Palmaria decipiens	Deception Island	S 62°58′788″ W 60°33′ 464″
Ramalina terebrata	Punta Hannah—Livingston Island	S 62°39´248″ W 60°36´701″
Usnea aurantiacoatra	Half Moon Island	S 62°35′511″ W 59°54′095″
Shells on bird´s nest	King George Island	S 62°05′948″ W 58°23′622″

K_2HPO_4 0.3 g, $MgSO_4 \times 7\ H_2O$ 0.05 g, Agar 15 g; Reasoner and Geldreich 1985), NA (Nutrient agar: Beef Extract 3 g, Peptone 5 g, Agar 15 g), and TSA (Trypticase soy agar: tryptone 15 g, soya peptone 5 g, sodium chloride 5 g, Agar 15 g). Artificial seawater (ASW) was used for isolation of bacteria from marine samples and distillated water for terrestrial samples, both supplemented with cycloheximide (300 µg. mL^{-1}). R2A medium was used aiming to recover slow-growing bacterial species that would quickly be suppressed by fast-growing species on a richer culture medium (Margesin et al. 2012). Samples were pre-washed with sterile seawater to avoid the isolation of surface microorganisms and cut into small fragments, which were placed onto the culture media cited above. The plates were kept at 5 and 15 °C for 10 days. Bacterial growth was monitored every 24 h and colonies were transferred to the same medium used for isolation. All purified bacteria were preserved at − 80 °C in 20% glycerol in the research holding of the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI) at Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA) at the Campinas State University (UNICAMP). For bacterial identification, genomic DNA from the pure cultures was obtained according to the protocol described by (Pitcher et al. 1989). PCR amplification with 10f and 1100r primers (Lane 1991), sequencing and phylogenetic analyses of partial 16S rRNA gene fragments were carried out as described previously by Belgini et al. (2014). The Ribosomal Database Project (RDP) was used to select the most related species for further phylogenetic reconstruction.

Antimicrobial activity assay

Bacterial isolates obtained from Antarctica were tested as antimicrobial substance (AMS) producers using the agar diffusion method described by (Anthony et al. 1972). Bacteria were spot on NA-ASW agar and incubated at 15 °C for 7 days. After that, bacterial cells were killed by exposure to chloroform vapor during 15 min. After evaporation of residual chloroform, the plates were covered with a layer of semi-solid medium (0.5% agar) inoculated with one of the indicator strains: *E. coli* **ATCC 11775**, *Micrococcus luteus* **ATCC 4698**, *Staphylococcus aureus* **ATCC 6538**, *Bacillus subtilis* **ATCC 6051**, and *Candida albicans* **ATCC 10231.**

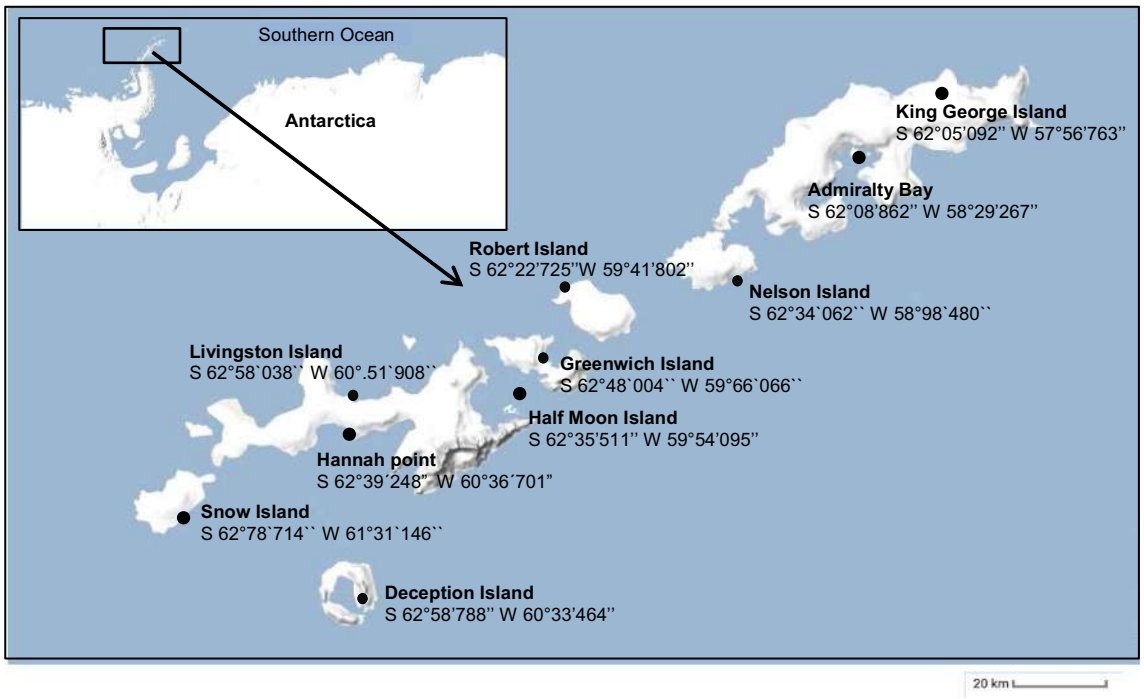

Fig. 1 Geographic location of sampling sites (●) in South Shetland Islands (Antarctica). (Color figure online)

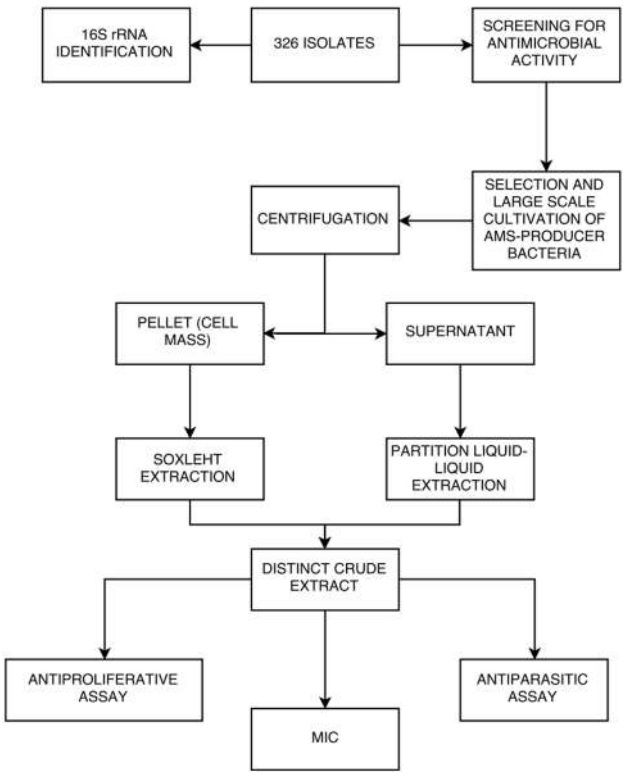

Fig. 2 Flowchart depicting the methodological strategy adopted in this work

Inhibition halo around the colony indicated the production of AMS.

Cultivation of AMS-producing bacteria

The selected AMS-producing bacterial isolate was grown in 5 L of TSB-ASW (Tryptic Soy Broth) for 72 h at 15 °C to produce biomass for subsequent chemical analyses. Cells were collected by successive centrifugations at 8.000×*g* (Eppendorf 5410) for 10 min at 4 °C and stored at 5 °C. Considering a possible accumulation of antimicrobial substance in the culture medium, the supernatant fraction was also separated for chemical analyses.

Soxhlet extraction

Soxhlet is a continuous solid/liquid extractor that allows an unmanaged and unmonitored operation while efficiently recycling a small amount of solvent (Jensen 2007; Luque de Castro and Priego-Capote 2010; Schmidt et al. 2014). Cell biomass from each pellet underwent a successive soxhlet extraction using an increasing polar gradient. First, around 25 g of cells were extracted with 200 mL of hexane until exhaustion (24 h). The crude hexane extract was removed from the soxhlet system and reserved in an Erlenmeyer flask. Cells were then subjected to another extraction with 200 mL of methanol for 24 h until completion. Crude extracts were concentrated under vacuum in a rotary evaporator at 40 °C

until complete dryness and weighed to calculate the yield of each extraction.

Liquid–liquid partition extraction

Partition is a method that separates compounds based on their relative solubility in two different immiscible liquids, the culture broth (polar) and ethyl acetate (non-polar) (Rezaee et al. 2006; Sant'Anna et al. 2016). Each portion of the microbial culture broth (1000 mL) was transferred to a 2000 mL separating funnel to be partitioned with Ethyl Acetate (500 mL, 3 times). After shaking, the organic phase was collected and treated with anhydrous sodium sulfate, followed by filtration. The extracts were then concentrated under vacuum and weighed for yield calculations (Harder et al. 2002).

Pharmacological assays for determining in vitro antiproliferative activity

This assay aimed to detect anticancer activities by evaluating antiproliferative action in human tumor cells (Monks et al. 1991). In vitro tests were performed with the crude extract of *Pseudomonas* sp. 99 on human tumor cell lines of different origins and characteristics, as follows: breast cancer (MCF-7), lung cancer (NCI-H460), and glioblastoma (U251) provided by the National Cancer Institute at Frederick MA-USA. Stock cultures were grown in complete medium [RPMI 1640 (GIBCO) supplemented with 5% fetal bovine serum (FBS, GIBCO) and 1% (v/v) penicillin:streptomycin (Nutricell, 1000 U/mL:1000 g/mL)] in a humidified atmosphere with 5% CO_2, at 37 °C. For the experiments, cell lines were used between passages 4–12. Cells in 96-well plates (100 µL cells/well) were exposed to different bacterial extract concentrations in dimethyl sulfoxide (DMSO)/RPMI (0.25, 2.5, 25, and 250 µg/mL) and incubated at 37 °C with 5% of CO_2 for 48 h. Final DMSO concentration did not affect cell viability. Doxorubicin chloride (Europharma, 0.025, 0.25, 2.5, and 25 µg/mL) was used as a positive control. Before (T0 plate) and after sample addition (T1 plates), cells were fixed with 50% trichloroacetic acid and cell growth determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B (SRB) assay. Three measurements were obtained: 1) time zero (T0, at the beginning of incubation); 2) 48 h post-incubation for compound free (C), and 3) tested (T) cells. Cell proliferation was determined according to the equation $100 \times \left[(T - T0)/C - T0\right]$, for $T0 < T \leq C$, and $100 \times \left[(T - T0)/T0\right]$, for $T \leq T0$. A concentration–response curve for each cell line was plotted using the software Origin 8.0 (OriginLab Corporation). From the concentration–response curve for each cell line, TGI (total growth inhibition or cytostatic effect) value was determined by non-linear regression analysis using the software Origin 8.0® (OriginLab Corporation) (Shoemaker 2006).

Antimicrobial activity by disk diffusion test and minimum inhibitory concentration (MIC)

The hexane extract of *Pseudomonas* sp. 99 (the selected AMS-producing bacterial isolate) was solubilized in 5% DMSO solution. Petri dishes containing Mueller Hinton medium were seeded with 100 µL of the indicator strain culture at 10^8 cells/mL, by the spread plate technique. Then 3 µL (1 mg) of the extract sample was spot shaped on the disk, followed by incubation at 35 °C. The absence of the indicator strain growth indicated the antimicrobial activity. MIC tests were carried out using a tissue culture test plate (96 wells) against the following strains: *Escherichia coli* ATCC 11775, *Micrococcus luteus* ATCC 4698, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6051, *Candida albicans* ATCC 10231. The stock solution of the extract was diluted and transferred into the first well, and serial dilutions were performed in order to obtain the concentration range of 1.6–0.012 mg/mL. The microbial inoculum was added to all wells and the plates were incubated at 37 °C during 24 h (bacteria) or at 30 °C for 48 h (yeast). Antimicrobial activity was detected by adding 20 µL of 0.5% TTC (triphenyl tetrazolium chloride, Merck) aqueous solution. MIC was defined as the lowest concentration that inhibited visible growth, as indicated by the TTC staining (Eloff 1998).

Antiparasitic assay

Trypanosoma cruzi assay was performed as described elsewhere (Moraes et al. 2014), using the Y strain. For the single-concentration primary screening, samples were tested at 10 µg/mL; 100 µg/mL was the highest concentration in the confirmatory dose–response assay, with ten 2-fold dilution points, and 104 µg/mL was the highest concentration for reference compound benznidazole. The Operetta high-content automated imaging system (Perkin Elmer) was used to acquire and analyze images. The analysis output was based on several parameters: host cell number, ratio of infected cells, and number of parasites per infected cell. The ratio of infected to total number of cells was then calculated, and defined as the Infection Ratio (IR). The raw data for IR values were normalized to negative—DMSO (mock)-treated infected cells—and positive (non-infected cells) controls to determine the normalized antiparasitic activity, expressed as a percentage of activity in comparison to wells. Sample cytotoxicity was determined by the cell ratio (number of cells in the test well divided by the average number of cells in negative control wells).

Results

Bacterial identification

From 600 bacterial isolates recovered from the Antarctic samples, 326 were identified based on the sequencing and phylogenetic analysis of the 16S rRNA gene. Marine sediments yielded a higher number of strains ($n = 109$), followed by biofilms ($n = 68$), bryozoans ($n = 48$), sponge ($n = 47$), soil ($n = 43$), penguin soil ($n = 7$), and lichen ($n = 4$) (Table 2). They were distributed in four phyla Actinobacteria (115 isolates, 35%), Proteobacteria (153 isolates, 47%), Bacteroidetes (31 isolates, 10%), and Firmicutes (28 isolates, 8%) (Table 2).

Phylogenetic analysis, using the RDP tool and Bayesian classifier to select the closest related strains, permitted the identification of many bacterial isolates at the species level as shown in the phylogenetic trees (Electronic Supplementary Material—ESM). Species of the Alphaproteobacteria class (Online Resource 1) were identified as *Sulfitobacter litoralis* ($n = 8$), *Sulfitobacter donghicola* ($n = 4$), and *Loktanella salsilacus* ($n = 2$). The Betaproteobacteria class accounted with only one species, *Polaromonas hydrogenivorans* ($n = 1$). The Gammaproteobacteria group (Online Resource 2) was the most represented among the bacteria cultivated from the Antarctic samples, and thus isolates were identified based on three different phylogenetic trees: one for *Pseudoalteromonas* genus (Online Resource 3), one for *Psychrobacter* genus (Online Resource 4), and one for the other genera. Members of *Psychrobacter* genus were identified as follows: *P. urativorans* ($n = 1$), *P. cibarius* ($n = 4$), *P. cryohalolentis* ($n = 4$), and *P. maritimus* ($n = 2$). *Pseudoalteromonas* members could not be identified at the species level, probably because the 16S rRNA gene is not an appropriate phylogenetic marker for this group. The other Gammaproteobacteria species recovered were *Pseudomonas frederikbergensis* ($n = 1$), *Marinomonas primoryensis* ($n = 10$), *Marinobacterium rhizophilum* ($n = 1$), *Marinobacter psychrophilus* ($n = 1$), and *Psychromonas arctica* ($n = 11$).

Actinobacteria members were identified based on two phylogenetic trees: one for *Arthrobacter* genus (Online Resource 5) and one for the remaining genera (Online Resource 6). The Actinobacteria species recovered (bootstrap > 97%) were *Leifsonia Antarctica* ($n = 3$), *Microbacterium testaceum* ($n = 5$), *Williamsia maris* ($n = 1$), and *Tomitella biformata* ($n = 1$). *Arthrobacter* species recovered were *Arthrobacter cryotolerans* ($n = 4$) and *Arthrobacter agilis* ($n = 2$).

Species belonging to Firmicutes (Online Resource 7) (bootstrap > 72%) were *Sporosarcina newyorkensis* ($n = 2$), *Sporosarcina aquimarina* ($n = 1$), *Planococcus kocurii* ($n = 1$), *Brevibacillus agri* ($n = 1$), and *Paenibacillus lautus* ($n = 1$). Bacteroidetes species recovered (Online Resource 8) (bootstrap > 88%) were *Maribacter arcticus* ($n = 3$), *Cellulophaga fucicola* ($n = 4$), *Cellulophaga algicola* ($n = 1$), *Bizionia argentinensis* ($n = 2$), *Psychroserpens mesophilus* ($n = 1$), *Polaribacter sejongensis* ($n = 2$), *Winogradskyella damuponensis* ($n = 2$), *Winogradskyella eximia* ($n = 1$), and *Cyclobacterium qasimii* ($n = 2$).

Screening for antimicrobial activities

Bacterial isolates were tested in an antagonism assay against Gram-negative, Gram-positive, and yeast indicator strains: *Escherichia coli* ATCC 11775, *Micrococcus luteus* ATCC 4698, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6051, and *Candida albicans* ATCC 10231. Among the 600 bacterial strains recovered from the Antarctic samples, 15 were positive against, at least, one indicator strain. The antimicrobial activity, taxonomic affiliation, and inhibition halo size of isolates are provided in Table 3. *Bacillus safensis* SG-32 and *Streptomyces* B-131, previously known as antimicrobial-producing strains, were used as positive controls (C +). Seven strains (*Bacillus safensis* H12, *Sulfitobacter litoralis* 103, *Pseudomonas* sp. 99, *Bacillus* sp. 98, *Tsukamurella* sp. 216, *Cyclobacterium qasimii* 172, and *Pseudomonas frederiksbergensis* 631) showed broad antibacterial spectrum activity against Gram-negative and Gram-positive target bacteria. Only three bacterial strains (*Cellulophaga fucicola* 418, *Arthrobacter* sp. 423, and *Pseudoalteromonas* sp. 485) showed inhibitory activity against *Candida albicans.*

Bacteria which presented the largest inhibition halos (++ or +++) had the inhibitory activity confirmed in another antagonism assay. Five of them (*Bacillus* sp. 98, *Bacillus safensis* H12, *Pseudomonas* sp. 99, *Cyclobacterium qasimii* 172, and *Pseudomonas frederiksbergensis* 631) presented a significant inhibitory halo and were selected for further MIC experiment (Table 4).

Three strains (H12, 99, and 631) presented satisfactory MIC activity. *B. safensis* H12 showed MIC of 1.6 mg/mL against *E. coli* and *M. luteus*, 0.4 mg/mL against *S. aureus,* and 0.8 mg/mL against *B. subtilis*. *Pseudomonas* sp. 99 showed the lowest values of MIC, 0.1 mg/mL against *E. coli*, and 0.2 mg/mL against *S. aureus*. Finally, *P. frederiksbergensis* 631 presented MIC of 0.25 mg/mL against *S. aureus*. No extract exhibited inhibition against *C. albicans.* Due to the lowest values observed in the MIC experiment, *Pseudomonas* sp. 99 was selected for extract preparation and further biological assays.

Table 2 Identification of Antarctic bacterial isolates and their respective origin samples

Isolates	RDP best hit Bayesian classifier	Samples							
		Sediment	Biofilm	Penguin soil	Soil	Sponge	Bryozoans	Lichen	Total
	Actinobacteria								115
50cyt, 49cyt	*Arthrobacter agilis*	–	–	–	2	–	–	–	2
616, 505, 577, 599	*Arthrobacter cryotolerans*	2	–	–	–	1	1	–	4
595, 583, 493, 491, 228, 226, 112, 441, 490, 288, 291, 297, 385, 496, 298, 592, 443, 504, 591, 553, 346, 261, 293, 511, 259, 263, 366, 382, 586, 264, 549, 546, 608, 644, 345, 255, 455, 355, 356, 289, 620, 588, 587, 492, 469, 450, 430, 429, 408, 358, 296, 110, 558, 438, 437, 235, 56, 47, 51, 52, 83, 614, 97, 221, 265, 322, 407, 410, 473, 532, 540, 575, 597, 454, 576,	*Arthrobacter* sp.	21	29	–	12	5	8	1	76
560, 601, 453	*Cryobacterium* sp.	–	–	–	–	–	2	1	3
312, 458	*Curtobacterium* sp.	–	–	–	–	–	2	–	2
268, 254, 257	*Leifsonia antarctica*	–	3	–	–	–	–	–	3
222, 58, 61, 64, 205, 206	*Microbacterium testaceum*	–	6	–	–	–	–	–	6
253, 255, 266, 267, 403, 295, 198, 417, 426, 544, 547, 578, 639	*Rhodococcus* sp.	1	5	–	6	–	1	–	13
613, 596	*Salinibacterium amurskyense*	2	–	–	–	–	–	–	2
256	*Tomitella biformata*	–	1	–	–	–	–	–	1
216	*Tsukamurella* sp.	1	–	–	–	–	–	–	1
594	Unaffiliated Actinomycetales	–	1	–	–	–	–	–	1
643	*Williamsia maris*	–	–	–	1	–	–	–	1
	Alphaproteobacteria	–	–	–	–	–	–	–	19
305, 610	*Loktanella salsilacus*	1	1	–	–	–	–	–	2
129	*Roseovarius halocynthiae*	1	–	–	–	–	–	–	1
161, 310, 119	*Sphingopyxis* sp.	3	–	–	–	–	–	–	3
229, 100, 103, 115, 117, 127, 231, 240	*Sulfitobacter litoralis*	7	1	–	–	–	–	–	8
104, 273, 392, 393	*Sulfitobacter donghicola*	3	1	–	–	–	–	–	4
128	Unaffiliated Alphaproteobacteria	1	–	–	–	–	–	–	1
	Betaproteobacteria	–	–	–	–	–	–	–	1
P2-leo	*Polaromonas hydrogenivorans*	–	–	1	–	–	–	–	1
	Gammaproteobacteria	–	–	–	–	–	–	–	133
201	*Marinobacter psychrophilus*	1	–	–	–	–	–	–	1
95	*Marinobacterium rhizophilum*	1	–	–	–	–	–	–	1

Table 2 (continued)

Isolates	RDP best hit Bayesian classifier	Samples							
		Sediment	Biofilm	Penguin soil	Soil	Sponge	Bryozoans	Lichen	Total
477, 90, 124, 153, 156, 158, 246, 247, 299, 300	*Marinomonas primoryensis*	4	5	–	–	–	1	–	10
gosma	*Pantoea* sp.	–	–	–	1	–	–	–	1
195, 169, 244, eshm1-20, 563, 638, H, 510, 466, es1ph2-1, I, 640, 433, esph2-3, 274, 602, es1-9, 579, es8, 77, 71, es2-3, eshm2-1, 69, A, rg22, 641, es15, 126, es12, es1-19, es1-26, 76, es2-17, 279, 624, 555, 637	*Pseudoalteromonas* sp.	12	–	–	2	19	4	1	38
478, 600, 629, 99-b, 92, 99, 436, 163	*Pseudomonas* sp.	5	–	–	–	–	3	–	8
631	*Pseudomonas frederiksbergensis*	–	–	–	–	–	1	–	1
272, 422, 29r2a, 28r2a, 414, 30, 642, 619, 521, 456, 525, 26, 615, 598, 521, 31, 29, 475, 476, 445, 108, 11, 276, 148, 188, 219, 497, 290, 523, 527, 409, 424-2, 245, 79, 212, 269, 281, 514, 567, 270, 561, 283, 514, 515, r2a27, 60, 258, 470, 538, 552, 236	*Psychrobacter* sp.	15	8	–	11	2	15	–	51
566, 564, 565, 284	*Psychrobacter cibarius*	1	–	–	–	–	3	–	4
470, 538, 552, 236	*Psychrobacter cryohalolentis*	–	2	–	–	–	1	1	4
524, 519	*Psychrobacter maritimus*	–	–	–	2	–	–	–	2
494	*Psychrobacter urativorans*	–	1	–	–	–	–	–	1
251, es19, es2-11, es2-2-2, es23, es2-4, es2ph-10, esh2-38, eshm2-25, esph2-15, G	*Psychromonas arctica*	–	–	–	–	11	–	–	11
	Bacteroidetes								31
171, 397, 509	*Arenibacter* sp.	2	–	–	–	1	–	–	3
232, 107	*Bizionia argentinensis*	2	–	–	–	–	–	–	2
282	*Cellulophaga algicola*	1	–	–	–	–	–	–	1
416, 425, 418, 635	*Cellulophaga fucicola*	–	–	–	1	3	–	–	4
172, 215	*Cyclobacterium qasimii*	2	–	–	–	–	–	–	2
242, 132, 121	*Flavobacterium* sp.	3	–	–	–	–	–	–	3
130, 131, 173	*Maribacter arcticus*	3	–	–	–	–	–	–	3
271, 243	*Polaribacter sejongensis*	2	–	–	–	–	–	–	2
280	*Psychroserpens mesophilus*	1	–	–	–	–	–	–	1
217	*Winogradskyella eximia*	1	–	–	–	–	–	–	1
518, 309	*Winogradskyella damuponensis*	–	–	–	–	2	–	–	2

Table 2 (continued)

Isolates	RDP best hit Bayesian classifier	Samples							
		Sediment	Biofilm	Penguin soil	Soil	Sponge	Bryozoans	Lichen	Total
465, 118, 120, 168, 224, 395, 457	*Zobellia* sp.	4	–	–	–	3	–	–	7
	Firmicutes								28
391, ant19-4, 98, 98rug	*Bacillus* sp.	3	–	1	–	–	–	–	4
ant21-27	*Brevibacillus agri*	–	–	1	–	–	–	–	1
439, 406, 412, 474, 541, 93, 449, 413, 427, 387, 362	*Carnobacterium* sp.	1	3	–	1	–	6	–	11
122	*Paenibacillus lautus*	1	–	–	–	–	–	–	1
481	*Planococcus kocurii*	–	1	–	–	–	–	–	1
106, 96	*Planococcus* sp.	2	–	–	–	–	–	–	2
442	*Sporosarcina aquimarina*	–	–	–	1	–	–	–	1
48, 444, 550, ant5-9, ant17-4	*Sporosarcina* sp.	–	–	2	3	–	–	–	5
ant9-18, ant10-11	*Sporosarcina newyorkensis*	–	–	2	–	–	–	–	2
	No. of isolates	109	68	7	43	47	48	4	326

Pseudomonas sp. 99 extract preparation and biological activity evaluation

After proliferation, *Pseudomonas* sp. 99 biomass was separated by centrifugation from culture broth for further extraction. Biomass (25 g) extraction provided hexane (99 Hex, 99.8 mg) and methanol (99 Met, 386 mg) extracts while an ethyl acetate (99 Acet, 484 mg) extract was obtained from the culture broth (5 L). Results from the disk diffusion antimicrobial assay showed that all extracts inhibited *S. aureus* growth while 99 Acet and 99 Hex also inhibited *M. luteus* (Table 5).

The antiproliferative activity of the three extracts against three human tumor cell lines was expressed as TGI (Total growth inhibition—concentration that inhibited cell growth by 100%) (Table 6). According to Fouche et al. (2008), TGI values higher than 50 µg/mL represent inactive samples. This way, all extracts should be considered inactive however *Pseudomonas* sp. 99 hexane extract (99 Hex) seemed to contain antiproliferative substances in lower concentration as 99 Hex was able to slightly inhibit U251 (TGI = 81.24 µg/mL) and NCI-H460 (TGI = 98.29 µg/mL).

The three *Pseudomonas* sp. 99 extracts, at the single concentration of 10 µg/mL, were evaluated against *T. cruzi* Y strain, while the reference drug (positive control) benznidazole was tested at 104 µg/mL (400 µM) (Fig. 3). Again, *Pseudomonas* sp. 99 Hex was considered sufficiently active for further testing in dose–response as it presented normalized antiparasitic activity superior to 50% and host cell toxicity lower than 50%, while the other extracts 99 Met and 99 Acet were inactive. When tested in dose–response, sample 99-Hex presented only moderate anti-*T. cruzi* activity, with a maximum activity of 23.4%.

Discussion

Cultivated bacterial diversity from Antarctic samples

The psychrophilic bacteria isolated from different sites from the South Shetland Islands in Antarctica were taxonomically characterized based on 16S rRNA gene sequencing and phylogenetic analysis. Results showed that isolates were assigned to the major phylogenetic groups: Gammaproteobacteria (40%), Actinobacteria (35%), Bacteroidetes (10%), Firmicutes (8%), and Alphaproteobacteria and Betaproteobacteria (6%). The results obtained are corroborated by previous studies on microbial communities from various Antarctic habitats regardless of whether molecular or cultivation-based approaches were applied

Table 3 Inhibition results exhibited by Antarctic isolates against the indicator strains tested

AMS producer isolates		Indicator strains				
		M. luteus	*E. coli*	*B. subtilis*	*S. aureus*	*C. albicans*
H12	*Bacillus safensis*	++	+++	–	+++	–
201	*Marinobacter psychrophilus*	++	–	–	–	–
103	*Sulfitobacter litoralis*	+	+	–	++	–
117	*Sulfitobacter litoralis*	+	–	–	+	–
127	*Sulfitobacter litoralis*	+	–	–	++	–
99	*Pseudomonas* sp.	++	++	–	+++	–
98	*Bacillus* sp.	+++	+++	+	+++	–
132	*Flavobacterium* sp.	–	–	+	–	–
165	*Flavobacterium* sp.	–	–	+	–	–
216	*Tsukamurella* sp.	–	+	–	+	–
172	*Cyclobacterium qasimii*	++	++	+	+	–
418	*Cellulophaga fucicola*	–	–	–	–	+
423	*Arthrobacter* sp.	––	–	–	–	+
485	*Pseudoalteromonas* sp.	–	–	–	–	++
631	*Pseudomonas frederiksbergensis*	+	++	–	++	–
*SG-32	*Bacillus safensis* (C+)	+++	+++	+	+++	–
*B-131	*Streptomyces* sp. (C+)	–	+	–	–	+++

The absence of inhibition is represented by (–), (+) represents a slight/translucent inhibition halo of 4–10 mm diameter, (++) represents a moderate inhibition halo of 11–14 mm, and (+++) represents strong inhibition halos larger than 14 mm

*SG-32 and B-131 represent positive controls of the experiment

Table 4 Minimum inhibitory concentration (MIC) of bacterial extracts

Indicator strain	Producer isolates (mg/mL)				
	Bacillus sp. 98	*Bacillus safensis* H12	*Pseudomonas* sp. 99	*Cyclobacterium qasimii* 172	*Pseudomonas frederiksbergensis* 631
E coli ATCC 11775	–	1.6	0.1	–	–
M. luteus ATCC 4698	–	1.6	–	–	–
S. aureus ATCC6538	–	0.4	0.2	–	0.25
B. subtilis ATCC 6051	1.3	0.8	–	–	–
C. albicans ATCC 10231	–	–	–	–	–

The absence of inhibition is represented by (–)

(Bowman et al. 1997; Van Trappen et al. 2002; Wilmotte et al. 2012; Carr et al. 2013).

Thirty-nine bacterial genera were identified out of 326 isolates. Most of the strains were closely related to other known cultured bacteria.

Phylogenetic analyses showed that 45 strains remained unaffiliated (Online Resource), possibly representing new species, as follows: isolates 128 (closely related with *Antarctobacter heliothermus*), 629 (closely related with *Pseudomonas lutea*), 163 and 436 (*Pseudomonas* sp.), 445, 108, 111, 276, 148, 188, and 219 (closely related with *Psychrobacter luti*), 409, 424-2, 245, 79, 212, 269, 281, 514, 567, 270, 561, 283, 514, r2a27, 60, and 258 (closely related with *Psychrobacter cryohalolentis*), 594

Table 5 Evaluation of antimicrobial activity of *Pseudomonas* sp. 99 extracts by disk diffusion assay

Microorganism	Sample	Extract	Antibiogram	
			Micrococcus luteus	*Staphylococcus aureus*
Pseudomonas sp. 99	Culture broth	99 Acet	+	+
	Biomass	99 Hex	+	+
		99 Met	–	+

99 Hex Pseudomonas sp. 99 biomass hexane extract, *99 Met Pseudomonas* sp. 99 biomass methanol extract, *99 Acet Pseudomonas* sp. 99 broth ethyl acetate extract

Table 6 Antiproliferative activity of *Pseudomonas* sp. 99 extracts against human tumor cell lines

Sample	TGI (μg/mL)[b]		
	U251	MCF7	NCI-H460
[a]Doxorubicin	2.46	6.11	< 0.025
99 Hex	81.24	206.88	98.29
99 Met	> 250	> 250	> 250
99 Acet	> 250	> 250	> 250

Human tumor cell lines: U251 (glioma), MCF-7 (breast), NCI-H460 (lung)

99 Hex Pseudomonas sp. 99 biomass hexane extract, *99 Met Pseudomonas* sp. 99 biomass methanol extract, *99 Acet Pseudomonas* sp. 99 broth ethyl acetate extract

[a]Doxorubicin used as positive control

[b]Concentration that totally inhibited cell growth

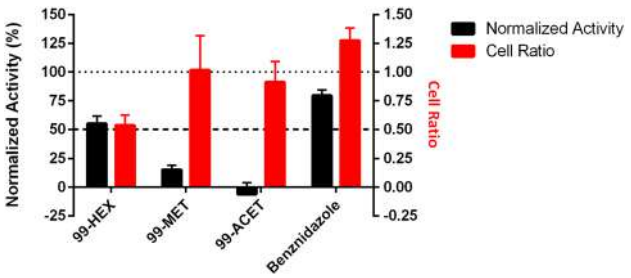

Fig. 3 Sample activity profile against U2OS cells infected with *T. cruzi* amastigotes: normalized activity (in %—black columns) and cell ratio (red columns). Error bar indicates standard deviation from two independent experiments. (Color figure online)

(closely related with *Leifsonia rubeus*), 595, 583, 493, 491, 228, 226, and 264 (*Arthrobacter* sp.), 171, 509, and 397 (*Arenibacter* sp.), 465, 118, 120, 168, 224, 395, and 457 (*Zobellia* sp). Four genera were shown to be predominant among the bacteria recovered from the Antarctic samples*: Arthrobacter* ($n = 82$), *Psychrobacter* ($n = 62$), *Pseudoalteromonas* ($n = 38$), and *Rhodococcus* ($n = 13$).

The genus *Arthrobacter* belongs to the Actinobacteria phylum and includes bacteria that are mostly mesophilic, with optimum growth below 30 °C, but some strains isolated from cold environments (Arctic, Antarctica, glaciers) are psychrotolerant or even psychrophilic (White et al. 2000; Pindi et al. 2010; Ganzert et al. 2011; Dsouza et al. 2015). Numerous *Arthrobacter* strains have been studied for the ability to degrade toxic compounds, such as 4-chlorophenol, 4-fluorophenol, 4-nitrophenol, or phenanthrene (Busse and Wieser 2014). Some strains were identified as a source of cold-adapted enzymes, such as chitinases and β-galactosidases (Lonhienne et al. 2001; Białkowska et al. 2009), and emulsifying agents (Rosenberg et al. 1979; Rosenberg et al. 2014). In this work 22 *Arthrobacter* sp. strains, more closely related to *A. psychrochitiniphilus/A. alpinus*, were accessed. *A. psychrochitiniphilus* strains are supposed to degrade chitin (Wang et al. 2009). Two strains were related to *Arthrobacter agilis*, a species associated with red carotenoid (bacterioruberin) production (Fong et al. 2001). *A. psychrophenolicus* has been described as degrading high phenol concentrations at low temperatures (Margesin et al. 2003).

Psychrobacter is an aerobic, osmotolerant, oxidase-positive, psychrophilic, or psychrotolerant bacterium. They occur in a wide range of wet, saline, and cold habitats (Dworkin 2006; Kim et al. 2012) but also in warm and slightly salty habitats. They have been studied for the production of cold-adapted enzymes such as β-lactamases (Feller et al. 1997). Isolation procedures used herein allowed the recovery of 62 isolates belonging to the *Psychrobacter* genus from Antarctic samples. However, identification of these isolates at the species level was not possible. According to Bakermans et al. (2006), *gyr*B gene represents a more reliable phylogenetic marker for the taxonomy of *Psychrobacter* species.

Pseudoalteromonas are marine bacteria, separated from the *Alteromonas* group in 1995 (Gauthier et al., 1995). They can be found in association with marine eukaryotes and may present antibacterial, bacteriolytic, agarolytic, and algicidal activities (Holmström et al. 1999). Taxonomic studies of *Pseudoalteromonas* group have revealed that 16S rRNA gene is highly conserved among the members of this taxon, not offering resolution for discriminating them at the species level. Romanenko et al. (2003) showed that *Pseudoalteromonas agarivorans* shared 99.9% 16S rRNA sequence similarity with *Pseudoalteromonas distincta, Pseudoalteromonas elyakovii, Pseudoalteromonas atlantica*, and *Pseudoalteromonas espejiana*. These reports are in accordance with the results observed in the phylogenetic reconstruction of the 38 isolates from Antarctic samples identified as *Pseudoalteromonas* sp.

Members of *Rhodococcus* genus are Gram-positive, aerobic, do not produce spore, and are closely related to *Mycobacterium* and *Corynebacterium*. Although some species are pathogenic, most are benign. They are found in many environments, including soil, water, and in association with eukaryotic cells (Finnerty 1992). The *Rhodococcus* members obtained in this study were mainly isolated from Antarctic biofilm and soil samples. Two species identified among the isolates, *R. kyotonensis* and *R. yunnanensis*, are associated to naphthalene-degrading activity (Anan`ina et al. 2011).

Antimicrobial-producing bacterial isolates

Biodiversity screenings, seeking for therapeutic and anti-tumor drugs from natural products, concentrates on metabolites with unusual properties. Consequently,

extremophiles, which developed biomolecules to thrive in particular living conditions, have been viewed as valuable sources of novel bioproducts, including antimicrobials (Cavicchioli et al. 2002; Sánchez et al. 2009). The antibiotic production of cold-loving organisms has not been investigated as extensively as those of the mesophiles (O'Brien et al. 2004). One reason may be the difficulty to detect psychrophilic microorganisms that produce antibiotics in cold environments. For example, Sánchez et al. (2009) recovered only 0.16% from 8,000 bacteria screened for antimicrobial activities, and O'Brien et al. (2004) detected 0.29% on their screening. However, studies focused on obtaining bacteria with antimicrobial activities from mesophilic environments identified much higher rates. For example, Romanenko et al. (2013) isolated 177 bacteria from deep-sea sediment in Japan and found that 13% of them were antimicrobial-producing bacteria when cultivated at 28 °C. Kennedy et al. (2009) isolated over 52 bacteria from a sponge at 28 °C and found that 50% had antimicrobial activity. In this study, 15 out of 600 (2.5%) bacterial isolates tested at 15 °C had the ability to inhibit the growth of the indicator strains used.

Some of the AMS-producing strains isolated in this work have been previously described in Antarctica, such as the genera *Arthrobacter, Pseudomonas* (Lo Giudice et al. 2007), *Flavobacterium*, *Marinobacter*, *Cyclobacterium*, and *Bacillus* (Rojas et al. 2009). Members of the genus *Tsukamurella* have been isolated from hydrothermal vents and reported as AMS producer (Eythorsdottir et al. 2016). Romanenko et al. (2013) described two *Sulfitobacter* strains that displayed antimicrobial activity, but no reports were found specifically for *Sulfitobacter litoralis,* that exhibited herein an antimicrobial inhibition against the Gram-positive bacteria *M. luteus* and *S. aureus*. Similarly, no reports were found about *Cellulophaga fucicola* as AMS producer.

Five strains *(Bacillus* sp. 98, *B. safensis* H12, *Pseudomonas* sp. 99, *Cyclobacterium qasimii* 172, and *P. frederiksbergensis* 631) stood out for the size and/or sharpness of the inhibition halo. It is worth noting that two other assays were previously performed, according to the methods described by (Engelhardt et al. 2010) and (Ichikawa et al. 1971) for the screening of antibiotic-producing bacteria. None of the two methodologies, however, resulted in clearly visible inhibition halos. The method that best suited was the one described by Anthony et al. (1972), in which colonies were first exposed to chloroform vapor prior to testing the growth of indicator strains.

The capacity of *Bacillus* species to produce substances with antimicrobial activity against a wide variety of microorganisms is well documented (Walker and Abraham 1970; Nakano and Zuber 1990; Leifert et al. 1995). Similarly, antimicrobial production by *Pseudomonas* sp. has been reported, including *P. frederiksbergensis* (Prasad et al. 2011; Melo et al. 2016). *Pseudomonas sp.* has already been described as cold active enzyme producer (Im et al. 2013), larvicidal activity (Mageswari et al. 2015), and poly-β-hydroxybutyrate producer (Li et al. 2013).

Pseudomonas sp. 99 was selected for further evaluation of its antiproliferative and antiparasitic potential due to a broad spectrum activity in MIC tests. Its extract (99 Hex) promoted growth inhibition of two tumor cell lines in concentration lower than 100 µg/mL. A pure extract compound of *Microbispora aerate*, isolated from penguin excrements in Antarctic Livingston Island, presented a low antiproliferative and cytotoxic effect in L-929 mouse fibroblast cells and K-562 human leukemia cells ($GI_{50} > 50$ µg/mL) (Ivanova et al. 2007; Ivanova et al. 2013). These results may suggest that *Pseudomonas* sp. 99-Hex extract could contain the active component of antiproliferative substances in low concentration, pointing out that a pure extract could be more effective against tumor cells. Changes in the culture parameters should afford better conditions for the production of antiproliferative compounds.

Little is known about the activity of antimicrobials against protozoan parasites (Vizioli and Salzet 2002). Assays indicate that antimicrobials could represent an effective appliance for the development of novel drugs to fight the parasite in the vertebrate host (Hancock 2000; Zasloff 2002). The antiparasitic test was performed against *T. cruzi*, the causative agent of Chagas disease or American trypanosomiasis, which is a chronic tropical infectious disease endemic in Latin America (World Health Organization 2013). The protozoan parasite *T. cruzi* occurs throughout the American continent and is transmitted by the triatomine bug insect vector, infecting a variety of mammals, including humans (Coura 2015). Antiparasitic activity of *Pseudomonas* sp. 99-Hex extract was sufficiently promising in primary screening at single concentration; however more studies are necessary for further confirmation.

Pseudomonas sp. 99 is closely related to *Pseudomonas* sp. BTN1 (Online Resource 9), isolated from Ross Sea sediment, Antarctica (Tedesco et al. 2016). This strain showed an antibacterial activity against different strains of the *Burkholderia cepacia* complex (Bcc) and Minimal Bactericidal Concentration (MBC) of 100 µg/mL against *S. aureus*, roughly similar to our result of 200 µg/mL in MIC test, considering that we tested the crude extract while Tedesco and co-workers tested the pure compound. Moreover, the authors achieved the isolation and identification of the antimicrobial compounds from *Pseudomonas* sp. BTN1, which corresponded to three rhamnolipids. Although the active compound in this study may not be same as the ones produced by *Pseudomonas* sp. BTN1, these results give us a clue on how to start the identification of the molecule (Tedesco et al. 2016).

Despite the great potential that cold environments hold for revealing diverse products and processes which may have numerous industrial applications, our knowledge from cold-adapted organisms remains limited. Moreover, as pharmaceutical concerns for new products arise, biological tools are increasingly replacing old means of processing materials and even show promise to meet societal needs. It is imperative that we continue investigating ways in which natural products can offer economic alternatives to the traditional processes (Margesin 2008).

In the present study, 326 bacteria were identified by 16S rRNA gene sequencing and 45 may represent unknown species. A deeper taxonomic survey is further necessary to fully characterize those isolates that were not clustered with recognized species in the phylogenetic trees (ESM). Results of antimicrobial, antiproliferative, and antiparasitic substance screening demonstrated that there is an untapped wealth in Antarctic environments for bioprospecting compounds with pharmaceutical potential application (O'Brien et al. 2004; Rojas et al. 2009). However, the small number of antibiotic-producing isolates obtained and the weakness of their inhibition halos corroborated previous findings suggesting that cold-loving bacteria from Antarctica are not as good as their relatives from mesophilic environments for antimicrobial prospecting (Kennedy et al. 2009; Romanenko et al. 2013). Nonetheless, antiproliferative and antiparasitic results observed are promising and further work is needed to elucidate the structure of the bioactive molecule produced by *Pseudomonas* sp. 99.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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Supplementary Material (ESM)

Bacteria from Antarctic environments: diversity and detection of antimicrobial, antiproliferative, and antiparasitic activities

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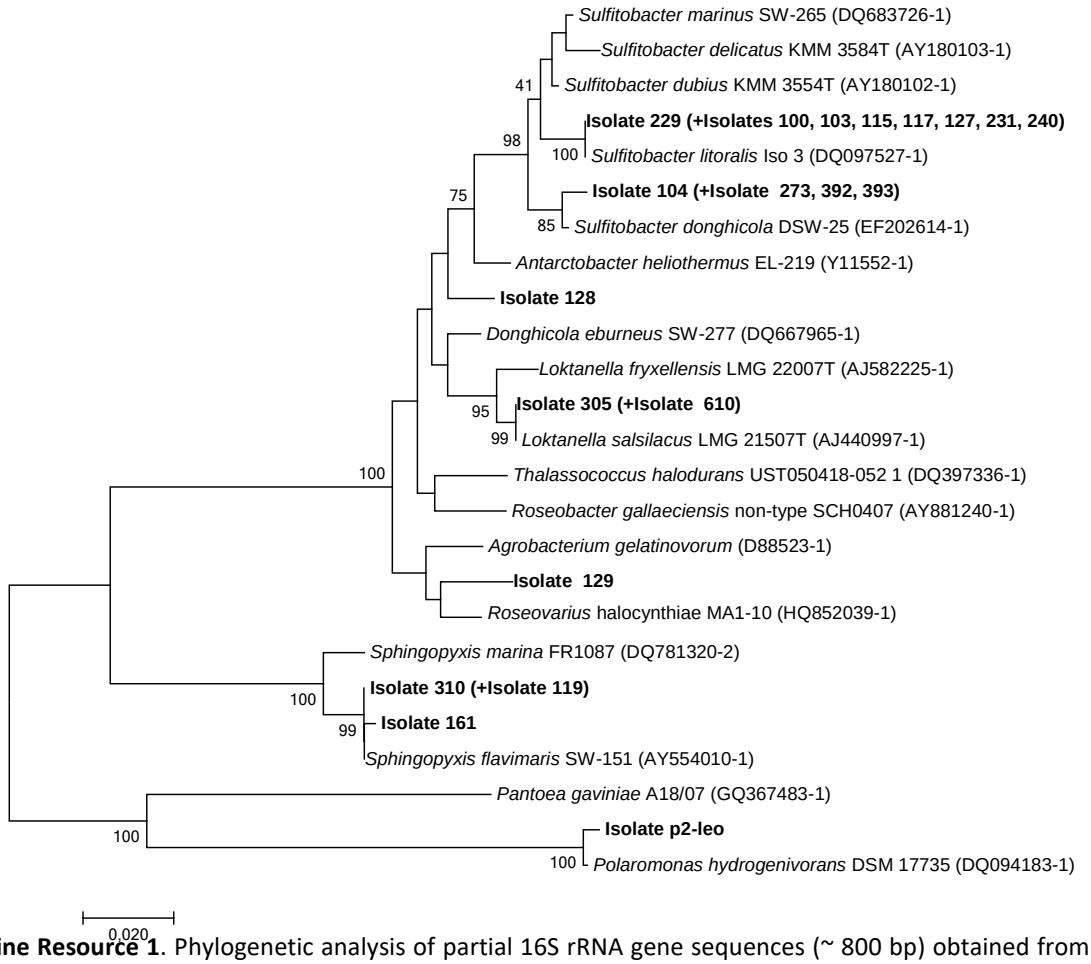
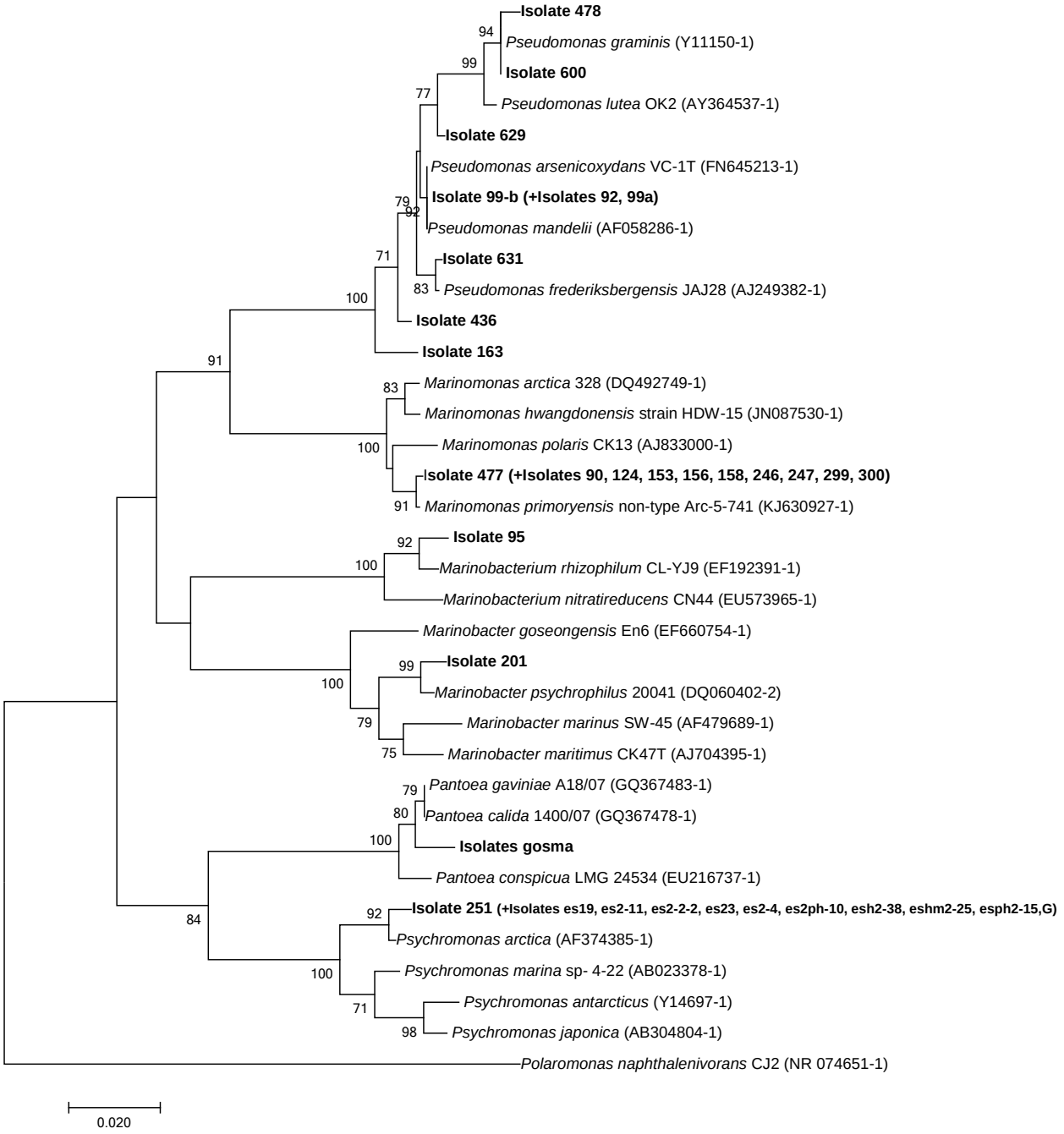
Polar Biology

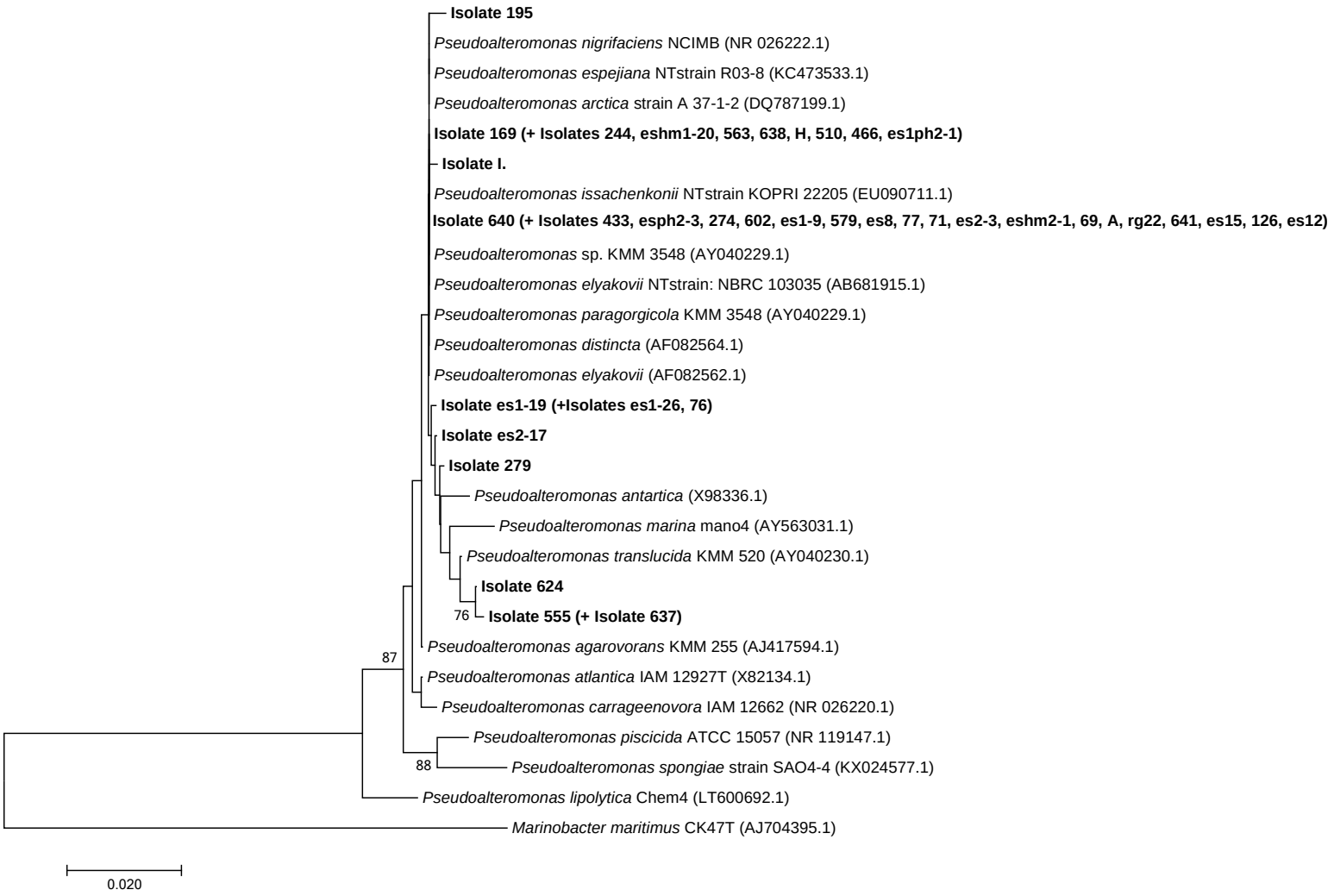
Phylogenetical tree of Antarctic isolates.



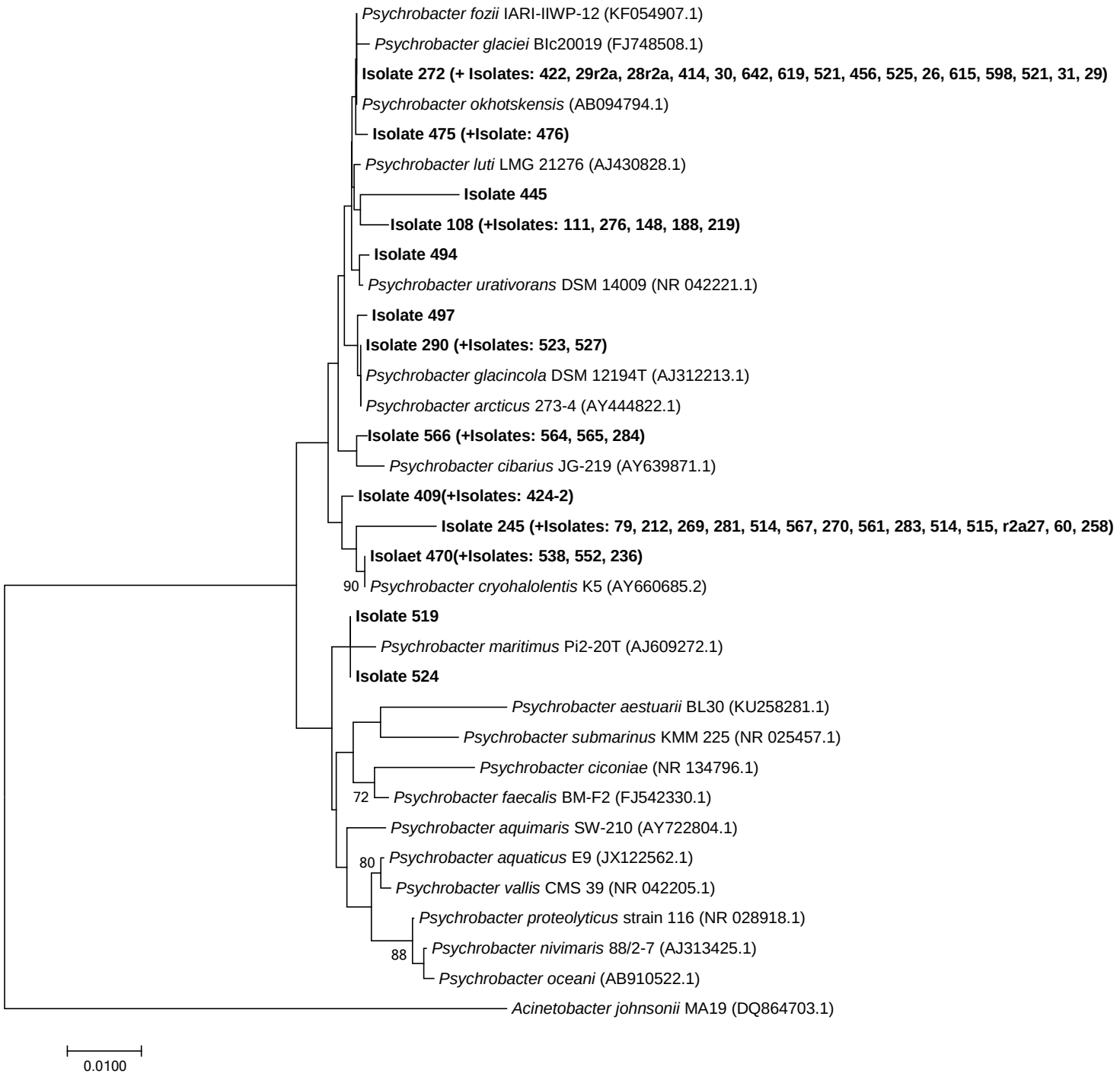
Online Resource 1. Phylogenetic analysis of partial 16S rRNA gene sequences (~ 800 bp) obtained from the bacterial isolates from Antarctic samples affiliated to **Alphaproteobacteria** and **Betaproteobacteria** related microorganisms retrieved from the RDP database. Evolutionary distance calculated with Kimura 2 parameters and phylogenetic tree made with the Neighbor-joining. Bootstrap values (1000 replicate runs shown as %) greater than 70% are listed



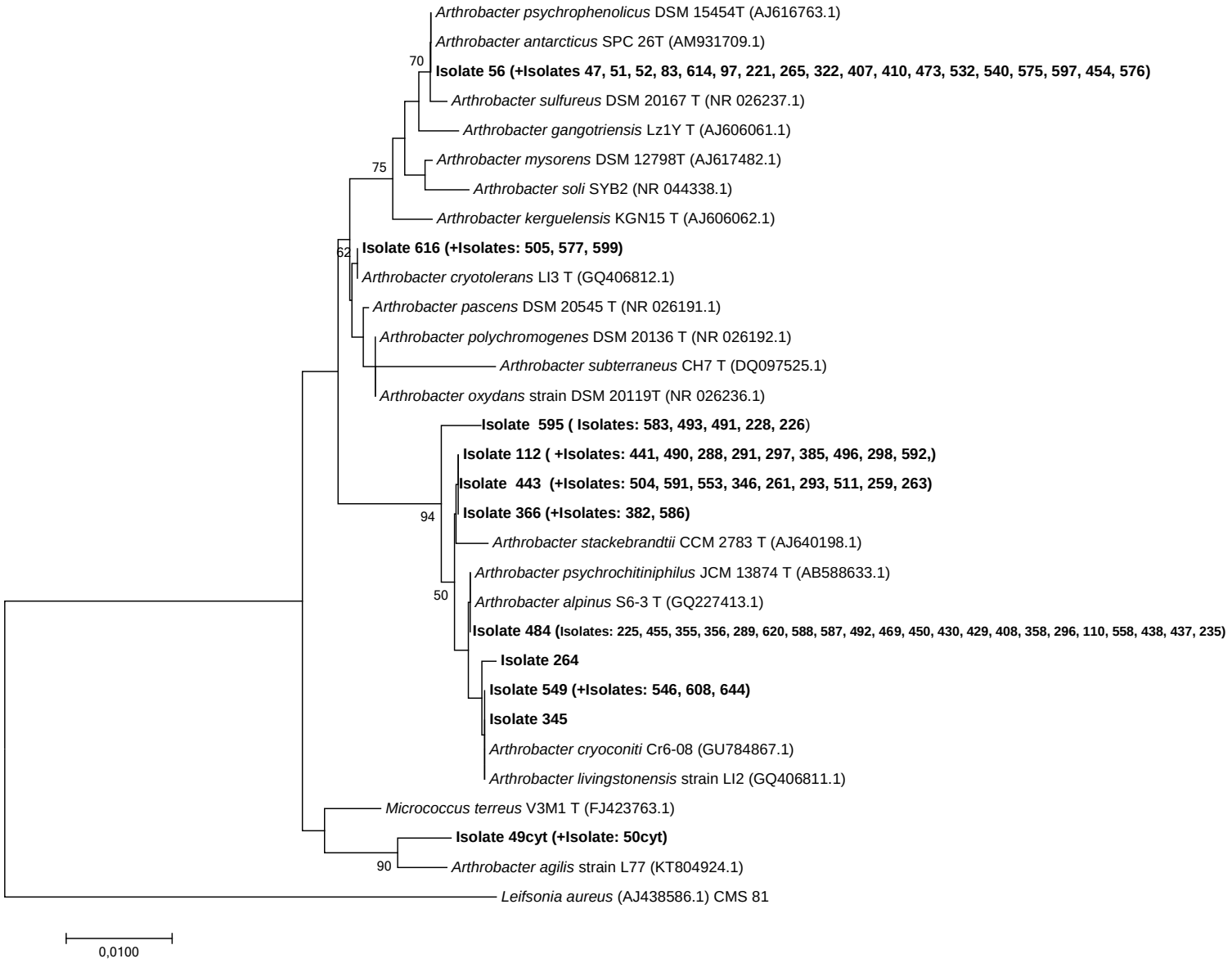
Online Resource 2. Phylogenetic analysis of partial 16S rRNA gene sequences (~ 800 bp) obtained from the bacterial isolates from Antarctic samples affiliated to **Gammaproteobacteria** and related microorganisms retrieved from the RDP database. Evolutionary distance calculated with Kimura 2 parameters and phylogenetic tree made with the Neighbor-joining. Bootstrap values (1000 replicate runs shown as %) greater than 70% are listed.



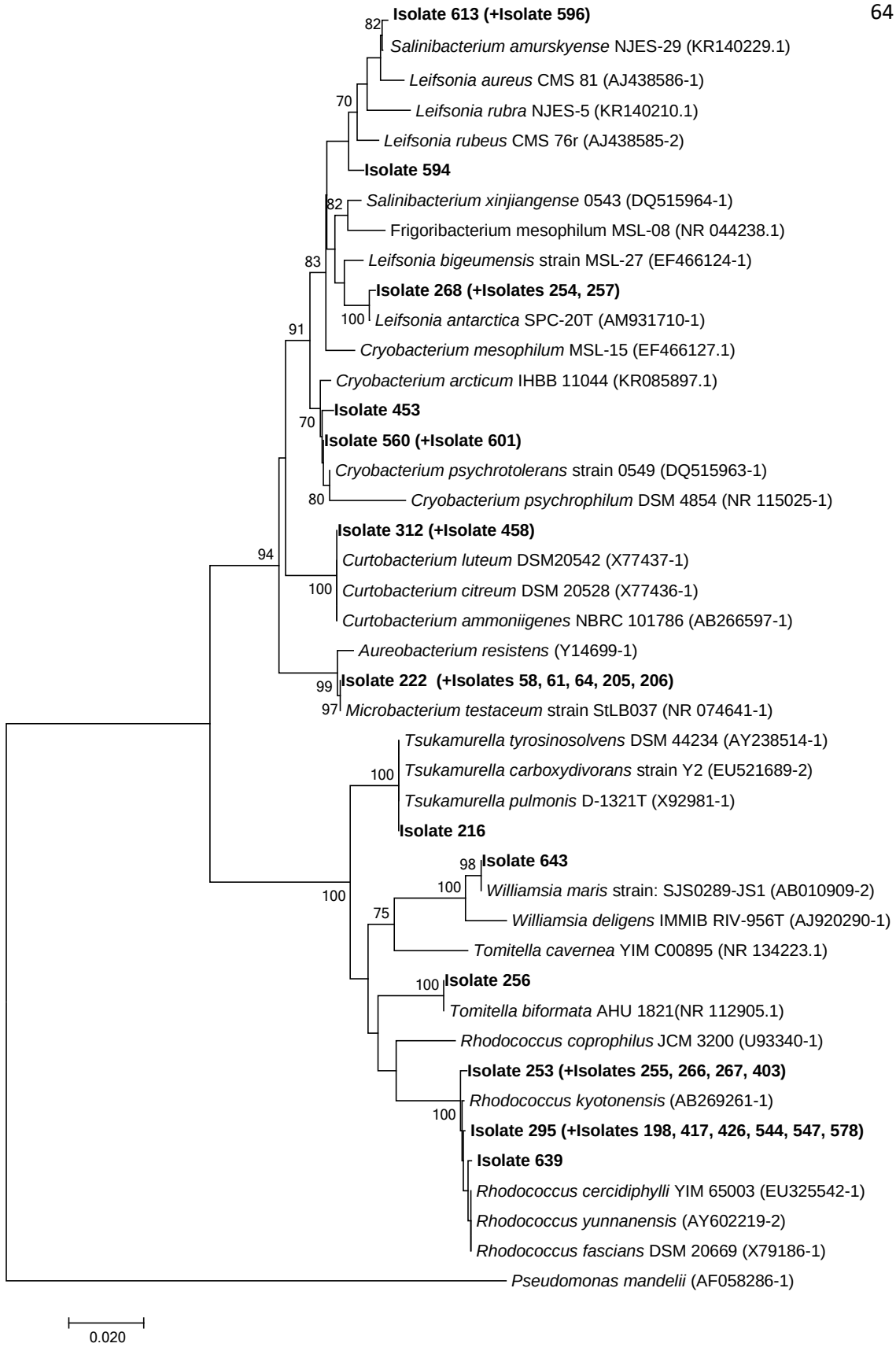
Online Resource 3. Phylogenetic analysis of partial 16S rRNA gene sequences (~ 800 bp) obtained from the bacterial isolates from Antarctic samples affiliated to **Pseudoalteromonas** and related microorganisms retrieved from the RDP database. Evolutionary distance calculated with Kimura 2 parameters and phylogenetic tree made with the Neighbor-joining. Bootstrap values (1000 replicate runs shown as %) greater than 70% are listed.



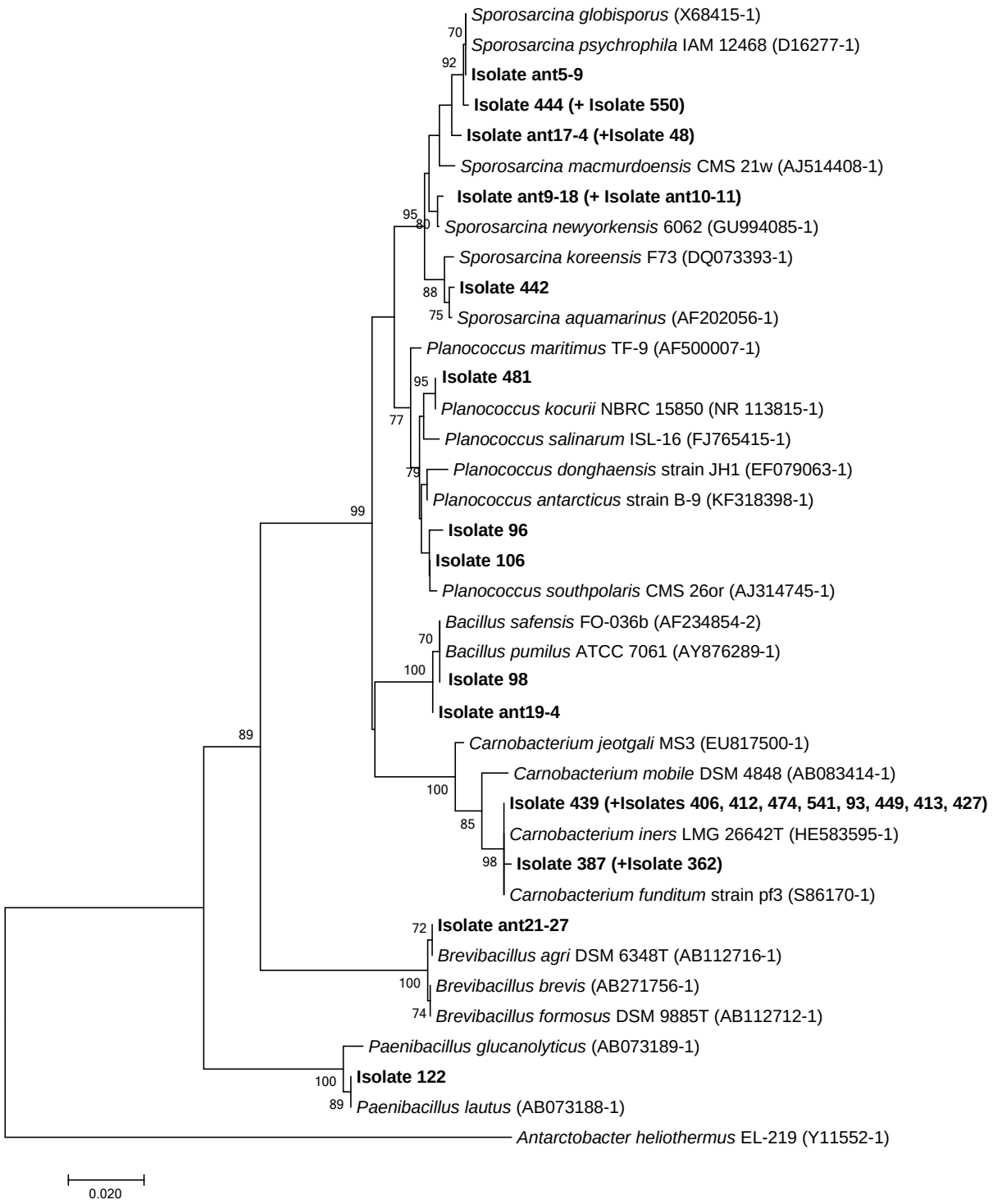
Online Resource 4. Phylogenetic analysis of partial 16S rRNA gene sequences (~ 800 bp) obtained from the bacterial isolates from Antarctic samples affiliated to **Psychrobacter** and related microorganisms retrieved from the RDP database. Evolutionary distance calculated with Kimura 2 parameters and phylogenetic tree made with the Neighbor-joining. Bootstrap values (1000 replicate runs shown as %) greater than 70% are listed.



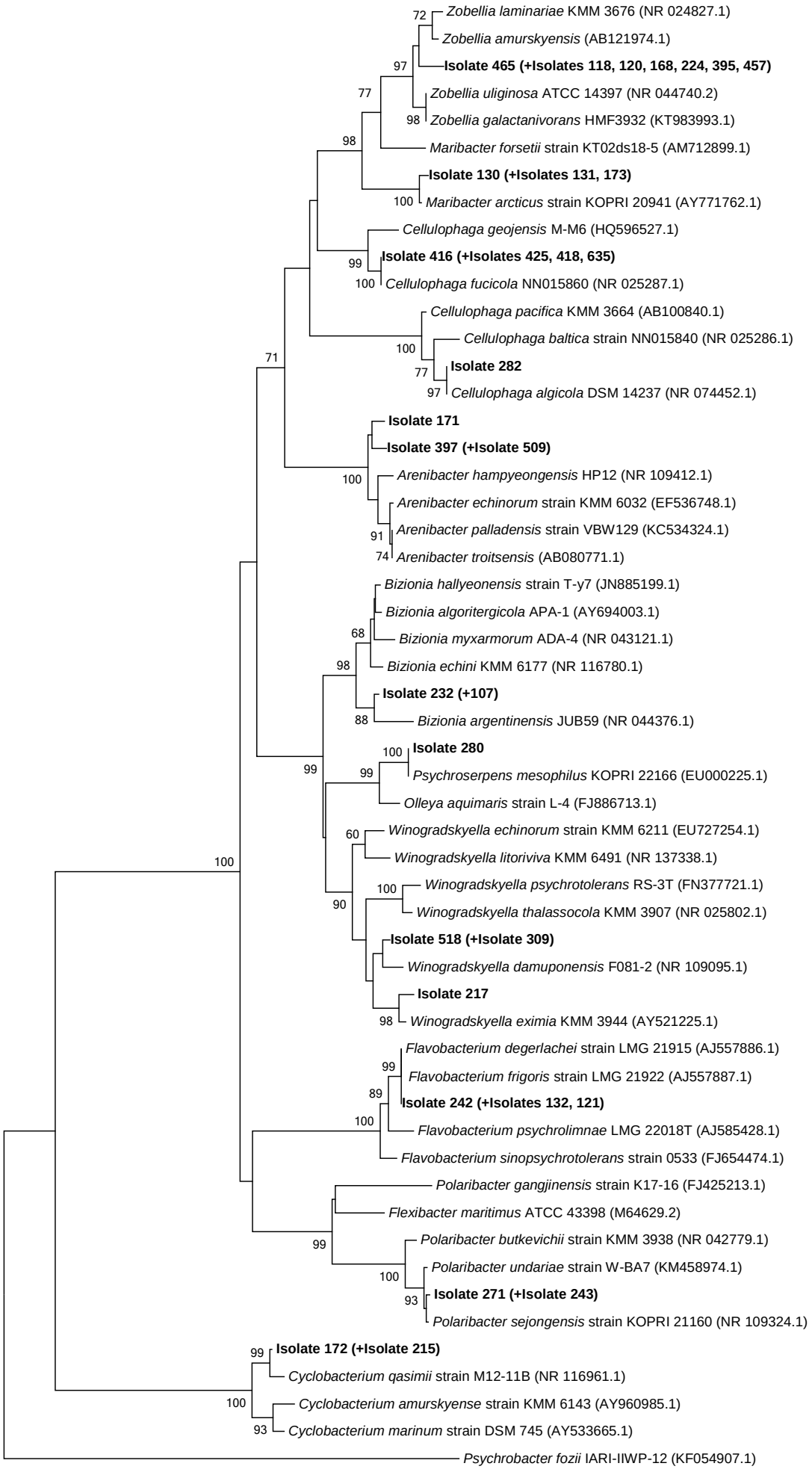
Online Resource 5. Phylogenetic analysis of partial 16S rRNA gene sequences (~ 800 bp) obtained from the bacterial isolates from Antarctic samples affiliated to **Arthrobacter** and related microorganisms retrieved from the RDP database. Evolutionary distance calculated with Kimura 2 parameters and phylogenetic tree made with the Neighbor-joining. Bootstrap values (1000 replicate runs shown as %) greater than 70% are listed.



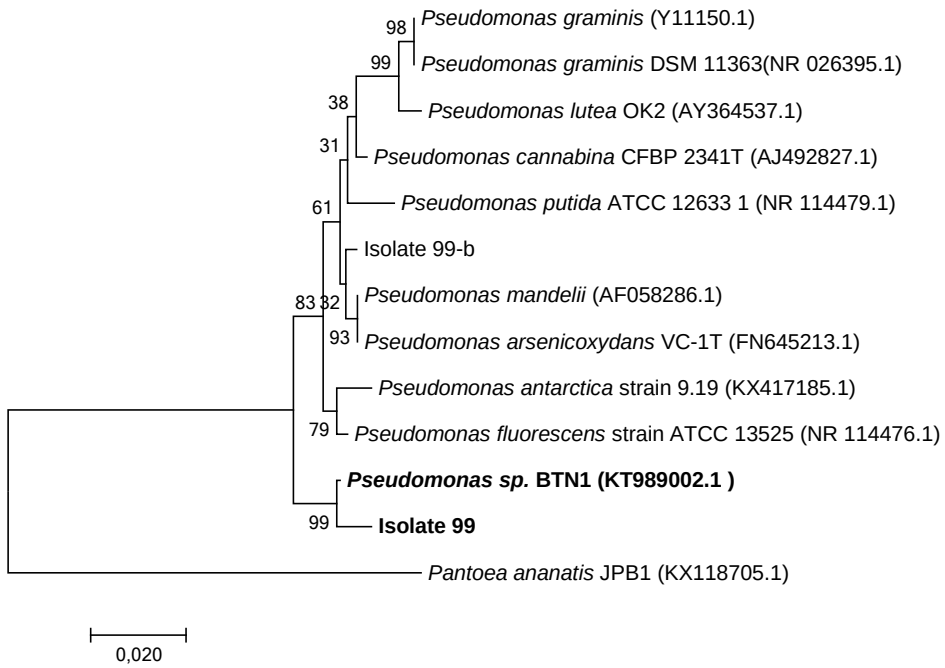
Online Resource 6. Phylogenetic analysis of partial 16S rRNA gene sequences (~ 800 bp) obtained from the bacterial isolates from Antarctic samples affiliated to **Actinobacteria** and related microorganisms retrieved from the RDP database. Evolutionary distance calculated with Kimura 2 parameters and phylogenetic tree made with the Neighbor-joining. Bootstrap values (1000 replicate runs shown as %) greater than 70% are listed.



Online Resource 7. Phylogenetic analysis of partial 16S rRNA gene sequences (~ 800 bp) obtained from the bacterial isolates from Antarctic samples affiliated to **Firmicutes** and related microorganisms retrieved from the RDP database. Evolutionary distance calculated with Kimura 2 parameters and phylogenetic tree made with the Neighbor-joining. Bootstrap values (1000 replicate runs shown as %) greater than 70% are listed.


Zobellia laminariae KMM 3676 (NR 024827.1)
Zobellia amurskyensis (AB121974.1)
Isolate 465 (+Isolates 118, 120, 168, 224, 395, 457)
Zobellia uliginosa ATCC 14397 (NR 044740.2)
Zobellia galactanivorans HMF3932 (KT983993.1)
Maribacter forsetii strain KT02ds18-5 (AM712899.1)
Isolate 130 (+Isolates 131, 173)
Maribacter arcticus strain KOPRI 20941 (AY771762.1)
Cellulophaga geojensis M-M6 (HQ596527.1)
Isolate 416 (+Isolates 425, 418, 635)
Cellulophaga fucicola NN015860 (NR 025287.1)
Cellulophaga pacifica KMM 3664 (AB100840.1)
Cellulophaga baltica strain NN015840 (NR 025286.1)
Isolate 282
Cellulophaga algicola DSM 14237 (NR 074452.1)
Isolate 171
Isolate 397 (+Isolate 509)
Arenibacter hampyeongensis HP12 (NR 109412.1)
Arenibacter echinorum strain KMM 6032 (EF536748.1)
Arenibacter palladensis strain VBW129 (KC534324.1)
Arenibacter troitsensis (AB080771.1)
Bizionia hallyeonensis strain T-y7 (JN885199.1)
Bizionia algoritergicola APA-1 (AY694003.1)
Bizionia myxarmorum ADA-4 (NR 043121.1)
Bizionia echini KMM 6177 (NR 116780.1)
Isolate 232 (+107)
Bizionia argentinensis JUB59 (NR 044376.1)
Isolate 280
Psychroserpens mesophilus KOPRI 22166 (EU000225.1)
Olleya aquimaris strain L-4 (FJ886713.1)
Winogradskyella echinorum strain KMM 6211 (EU727254.1)
Winogradskyella litoriviva KMM 6491 (NR 137338.1)
Winogradskyella psychrotolerans RS-3T (FN377721.1)
Winogradskyella thalassocola KMM 3907 (NR 025802.1)
Isolate 518 (+Isolate 309)
Winogradskyella damuponensis F081-2 (NR 109095.1)
Isolate 217
Winogradskyella eximia KMM 3944 (AY521225.1)
Flavobacterium degerlachei strain LMG 21915 (AJ557886.1)
Flavobacterium frigoris strain LMG 21922 (AJ557887.1)
Isolate 242 (+Isolates 132, 121)
Flavobacterium psychrolimnae LMG 22018T (AJ585428.1)
Flavobacterium sinopsychrotolerans strain 0533 (FJ654474.1)
Polaribacter gangjinensis strain K17-16 (FJ425213.1)
Flexibacter maritimus ATCC 43398 (M64629.2)
Polaribacter butkevichii strain KMM 3938 (NR 042779.1)
Polaribacter undariae strain W-BA7 (KM458974.1)
Isolate 271 (+Isolate 243)
Polaribacter sejongensis strain KOPRI 21160 (NR 109324.1)
Isolate 172 (+Isolate 215)
Cyclobacterium qasimii strain M12-11B (NR 116961.1)
Cyclobacterium amurskyense strain KMM 6143 (AY960985.1)
Cyclobacterium marinum strain DSM 745 (AY533665.1)
Psychrobacter fozii IARI-IIWP-12 (KF054907.1)
0,050

Online Resource 8. Phylogenetic analysis of partial 16S rRNA gene sequences (~ 800 bp) obtained from the bacterial isolates from Antarctic samples affiliated to **Bacteroidetes** and related microorganisms retrieved from the RDP database. Evolutionary distance calculated with Kimura 2 parameters and phylogenetic tree made with the Neighbor-joining. Bootstrap values (1000 replicate runs shown as %) greater than 70% are listed.



Online Resource 9. Phylogenetic analysis of partial 16S rRNA gene sequences (~ 800 bp) obtained from the isolate 99 from Antarctic affiliated to ***Pseudomonas sp*. BTN1** and related microorganisms retrieved from the RDP database.

CAPÍTULO II

Chemical characterization and biotechnological applicability of pigments isolated from Antarctic bacteria

Caracterização química e aplicações biotecnológicas dos pigmentos isolados de bactérias da Antártica

Marine Biotechnology

Bacterial pigments isolated from Antarctica strains reveals potential biotechnological application as UV-protective

--Manuscript Draft--

Manuscript Number:	MBTE-D-18-00151
Full Title:	Bacterial pigments isolated from Antarctica strains reveals potential biotechnological application as UV-protective
Article Type:	Original Paper
Funding Information:	Fundação de Amparo à Pesquisa do Estado de São Paulo (2014/17936-1; 2016/05640-6; 2017/21790-0) — Dr. Tiago Silva
Abstract:	Considering the global trend in the search for alternative natural compounds with antioxidant and Sun Protector Factor (SPF) boosting properties, bacterial carotenoids represent an opportunity for exploring pigments of natural origin which possess high antioxidant activity, lower toxicity, no residues, no environmental risk and are readily decomposable. In this work, three pigmented bacteria from the Antarctic continent, named Arthrobacter agilis 50cyt, Zobellia laminarie 465 and Arthrobacter psychrochitiniphilus 366, were able to withstand UV-B and UV-C radiation. The pigments were extracted and tested for UV absorption, antioxidant capacity, photostability and phototoxicity profile in murine fibroblasts (3T3 NRU PT - OECD TG 432) to evaluate their further potential use as UV-filters. Furthermore, the pigments were identified by ultra-high-performance liquid chromatography - photodiode array detector - mass spectrometry (UPLC-PDA-MS/MS). The results showed that all pigments presented a very high antioxidant activity and good stability under exposure to UV light. However, except for a fraction of the A. agilis 50cyt pigment, they were shown to be phototoxic. A total of 18 different carotenoids were identified from 23 that were separated on a C18 column. The C50 carotenes bacterioruberin and decaprenoxanthin (including its variations) were confirmed for A. agilis 50cyt and A. psychrochitiniphilus 366, respectively. All-trans-bacterioruberin was identified as the pigment that did not express phototoxic activity in the 3T3 NRU PT assay (MPE<0.1). Zeaxanthin, β-cryptoxanthin, β-carotene and phytoene were detected in Z. laminarie 465. In conclusion, carotenoids identified in this work from Antarctic bacteria open perspectives for their further biotechnological application towards a more sustainable and environmentally friendly way of pigments exploitation.
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Author Comments:	The present work comprises the characterization of pigments produced by three bacteria from the Antarctic continent named Arthrobacter agilis 50cyt, Zobellia laminarie 465 and Arthrobacter psychrochitiniphilus 366, which withstand UV radiation. The pigments were also tested for UV absorption, antioxidant capacity; photostability and phototoxicity profile in murine fibroblasts (3T3 NRU PT - OECD TG 432) to evaluate their further potential use as UV-filters. It is important to mention that this is the first report to describe a photoactivity behavior of carotenoids from Antarctica, more specifically the description of all-trans-bacterioruberin which did not present any phototoxic activity, opening perspectives for future use as UV-filter. Moreover, we found a high antioxidant potential for the pigment that opens perspectives for food and feeds use. Finally, I would like to clarify that all named authors agree with the publication of the work and declare that there are no conflicts of interest. All prevailing local, national and international regulations and conventions, and common scientific ethical practices have been respected.
Suggested Reviewers:	Adriana Mercadante azmercadante@gmail.com Specialist in carotene identification
	Laurent Dufossé laurent.dufosse@univ-reunion.fr Works with carotenoids identification
	Simon Cutting s.cutting@rhul.ac.uk He works with carotenoids in Bacillus
	Wan Azlina Ahmad azlina@kimia.fs.utm.my Made a review named Bacterial pigments and their applications
	Nazia Mojib abej@uab.edu She studied pigments from Antarctica

Chemical characterization and biotechnological applicability of pigments isolated from Antarctic bacteria

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ABSTRACT

Considering the global trend in the search for alternative natural compounds with antioxidant and Sun Protection Factor (SPF) boosting properties, bacterial carotenoids represent an opportunity for exploring pigments of natural origin which possess high antioxidant activity, lower toxicity, no residues, no environmental risk and are readily decomposable. In this work, three pigmented bacteria from the Antarctic continent, named *Arthrobacter agilis* 50cyt, *Zobellia laminarie* 465 and *Arthrobacter psychrochitiniphilus* 366, were able to withstand UV-B and UV-C radiation. The pigments were extracted and tested for UV absorption, antioxidant capacity, photostability and phototoxicity profile in murine fibroblasts (3T3 NRU PT - OECD TG 432) to evaluate their further potential use as UV-filters. Furthermore, the pigments were identified by ultra-high-performance liquid chromatography - photodiode array detector - mass spectrometry (UPLC-PDA-MS/MS). The results showed that all pigments presented free radical scavenger activity and good stability under exposure to UV light. However, except for a fraction of the *A. agilis* 50cyt pigment, they were shown to be phototoxic. A total of 18 different carotenoids were identified from those 23 separated on a C18 column. The C50 carotenes bacterioruberin and decaprenoxanthin (including its variations) were confirmed for *A. agilis* 50cyt and *A. psychrochitiniphilus* 366, respectively. *All-trans*-bacterioruberin was identified as the pigment that did not express phototoxic activity in the 3T3 NRU PT assay (MPE<0.1). Zeaxanthin, β–cryptoxanthin, β–carotene and phytoene were detected in *Z. laminarie* 465. In conclusion, carotenoids identified in this work from Antarctic bacteria open perspectives for their further biotechnological application towards a more sustainable and environmentally friendly way of pigments exploitation.

KEYWORDS: Antarctic pigments, Psychrophilic bacteria; *Arthrobacter*; *Zobellia*; Antioxidant; Carotenoids.

1. INTRODUCTION

Microorganisms inhabiting the Antarctic continent need to survive in extreme environments, such as severe weather conditions, dramatically changing light conditions, water and nutrient deficit, and high seasonal ultraviolet radiation (UVR) incidence, increased by the ozone depletion over Antarctica (the ozone "hole") (Uchino et al. 1999; Margesin et al. 2008; Margesin and Miteva 2011).

The UVR is one of the most damaging forms of radiation that reach the surface of the Earth and is consequently a highly important regulator of organism survival and ecosystem balance (Cockell and Knowland 1999; Dieser et al. 2010). The short-wavelength radiation UV-C (100-280 nm) is more damaging to biological systems than longer wavelengths. UV-B radiation, defined as 280–320 nm, is energetically less harmful than UV-C. UV-A radiation (315–400 nm) is still less energetic than UV-B radiation (Roos and Vincent 1998; Cockell and Knowland 1999). UVR, and particularly the higher energy wavelengths, has a range of effects, including DNA damage in most organisms (Harm 1980; Karentz 1991). To confront the damage caused by UV radiation, microorganisms have a well-known DNA repair mechanism (Pettijohn and Hanawalt 1964; Horneck 1995), quenching toxic intermediates (Wynn-Williams and Edwards 2002) and employing UV absorbing compounds such as pigments (Cockell and Knowland 1999).

A molecule absorbing a specific wavelength can release the absorbed energy by several mechanisms. Ideally, it returns unaltered to the ground state by releasing excess energy in nonradiative vibrational relaxation (heat), or by radioactive processes (fluorescence or phosphorescence), thus achieving photostability (Caspar and Meyer 1983). Energy can also be dissipated being transferred to other molecules via photoreactors causing photosensitivity. In this case, structural modifications may occur, which may be reversible (cis/trans isomerization or keto-enol tautomerization) or by mesomeric effects (Nguyen et al. 2013).

Thus, before screening natural products as new candidates for photoprotection, the study of photostability is essential to evaluate the possible effects of UV absorption, which can be harmful to the skin by the generation of photoreaction when applied topically. Phototoxicity is defined as a toxic response of a substance used to the body that is induced or augmented after exposure to light

(OECD 2004). A major indication of the non-phototoxicity of a substance is the maintenance of the molecular structure after solar irradiation, i.e., photostability.

The high frequency of pigment production in recovered isolates from ice samples (Bowman et al. 1997), marine surface waters (Agogué et al. 2005; Stafsnes et al. 2010) or remote glacial areas (González-Toril et al. 2008), indicate the importance of pigmentation in the adaptation process to cold and high UV environments. Some pigments are extracellular UV-filters; others have quenching properties to dissipate excess energy from UV-B, which would alternatively generate toxic singlet oxygen; some absorb UV-B inside the cell before metabolically important molecules can be damaged (Wynn-Williams et al. 2001). Also, there can be a combination of pigments which individually would not provide adequate protection but together minimize UVR damage either on an individual cell or in a mutually terrestrial community (Wynn-Williams and Edwards 2002).

These particular traits revealed that the Antarctic environment represents a vast pool of pigmented microbial biodiversity and exploitable biotechnology. This untapped diversity has resulted in increasing interest of the scientific community in the study of UV resistant microorganisms, exploiting their ability to produce novel metabolites/compounds with potential biotechnological applications. Thus some alternative natural compounds, such as mycosporine aminoacids (MAAs) (Scherer et al. 1988 and Ehling-Schulz et al. 1997), scytonemin (Taylor et al. 2010), carotenoids (Jagannadham et al. 1996; Jagannadham et al. 2000), prodigiosin and violacein (Suryawanshi et al. 2015) that present antioxidant and Sun Protection Factor (SPF) boosting properties (Shaath 2007) have been studied in bacterial strains. In this context, this work aimed to identify the pigments produced by the yellow *Arthrobacter psychrochitiniphilus* 366, the orange *Zobellia laminarie* 465 and the red *Arthrobacter agilis* 50cyt bacteria previously isolated from Antarctic samples (Silva et al. 2018). Moreover, the investigation of these pigments revealed their photostability, phototoxicity and antioxidant properties and suggests their possible application in commercial sunscreens.

2. MATERIAL AND METHODS

The experimental strategy followed to achieve identification of the pigments and the biological assays is detailed in Fig 1.

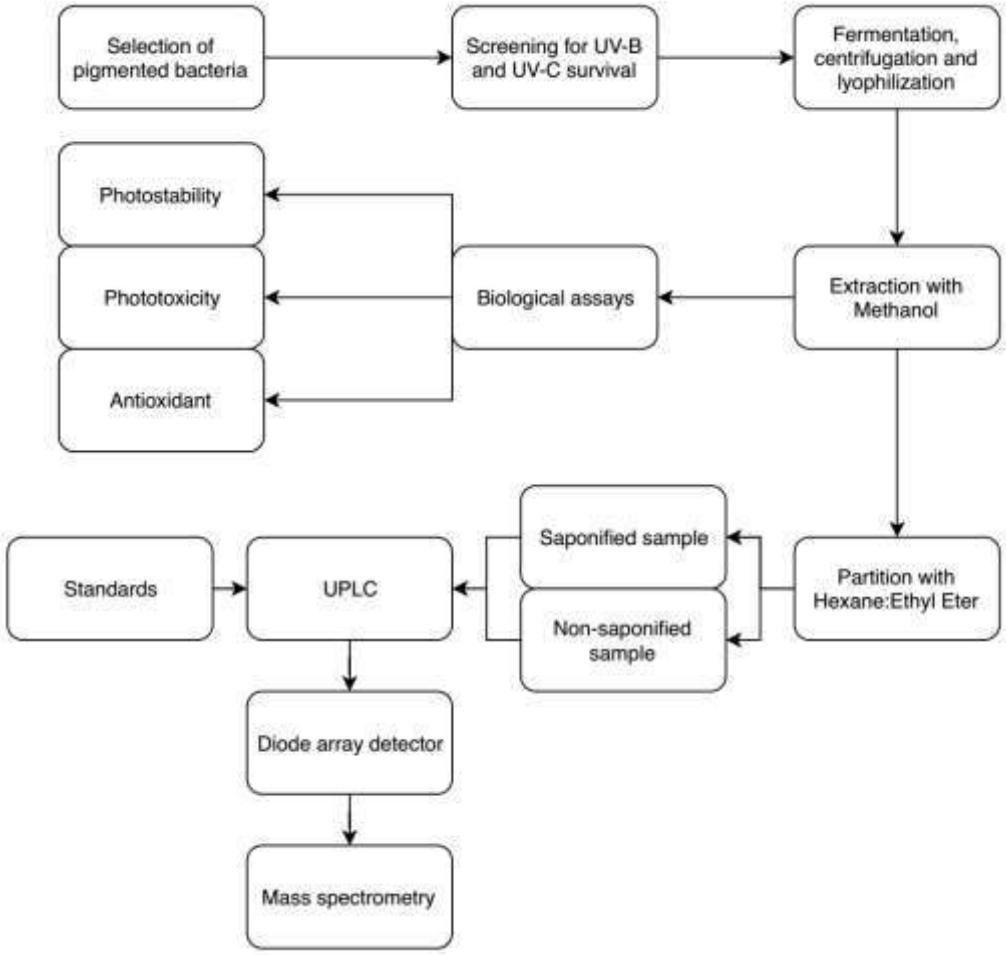


Fig 1. Workflow of the basic strategy to reach pigment identification.

2.1 Bacterial strains

The bacterial strain 50cyt was previously isolated from shells found on bird nest and identified as *A. agilis*. Strain 366 was isolated from defrost biofilm and identified as *A. psychrochitiniphilus*, and strain 465 was isolated from sea sponge and identified as *Z. laminarie.* All Antarctic samples used for isolating bacterial strains were collected during the expedition to maritime Antarctica in the Austral Summer (2013 and 2015) by the MycoAntar - Brazilian Antarctic Program team (Silva et al. 2018).

2.2 Chemicals

The standards β-carotene, β-cryptoxanthin, zeaxanthin, and astaxanthin were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Violaxanthin, canthaxanthin and antheraxanthin were purchased from Carotenature (Lupsingen, Switzerland). Methanol (MeOH), acetonitrile (ACN) and acetone (HPLC grade purity) were acquired from J.T. Baker (Deventer, The Netherlands). Water was purified in a Milli-Q reagent water system.

2.3 Bacterial cultivation

One hundred µL-aliquots of the bacterial cultures preserved in glycerol at -80^{o}C were inoculated into 300 mL of Nutrient Broth (NB) culture medium to be used as inoculum for the shake flasks and the fermentation. Cultivation for production of biomass for further pigment characterization was performed in 1 L-Erlenmeyer flasks filled with 600 mL NB medium diluted in artificial seawater (ASW). A total of 3000 mL of medium was used. Cultivation flasks were incubated for 7 days at 15°C in a rotary shaker operated at 100 rpm. Aliquots of 50 mL were harvested by centrifugation at 8000 × *g* and the cell pellet was washed with 10 mL of distilled water and recentrifuged. The cell pellet was freeze-dried and stored at -20°C prior to extraction of pigments.

2.4 Extraction of pigments and sample preparation

Lyophilized cells were extracted with 20 mL MeOH and vortexed for 5 min to ensure even dispersal of biomass. After centrifugation, the supernatant was collected, transferred to amber glass, concentrated under vacuum and the mass was measured. The crude extract was flushed with nitrogen and wrapped in aluminum foil to prevent light-mediated oxidation of the extracted carotenoids. Before analysis, the crude extract of pigment was suspended in 4 mL of hexane and shaken for 10 min. Next, 2.3 mL of a solution of NaCl (10%, w/v) was added. The mixture was shaken for 15 min, cooled at 4°C and the organic layer collected. The aqueous phase was re-extracted with 2 mL of hexane: diethyl ether (3:1, v/v) for 10 min and centrifuged at 2,500 x *g* for 4 min. The organic solutions were combined in a 10-mL glass tube and dried under nitrogen stream at room temperature (Delpino-Rius et al. 2014).

The average yield of crude pigment methanolic extract was about 50 mg/g for freeze-dried *A. agilis* 50cyt and *A. psychrochitiniphilus* 366, and 100 mg/g for *Z. laminarie* 465. After purification using semi preparative – MPLC, the yield of pigments was reduced to 1.8 mg/g of freeze-dried *A. agilis* 50cyt, 1.26 mg/g of *A. psychrochitiniphilus* 366 and 0.45 mg/g of *Z. laminarie* 465.

2.5 Saponification

An aliquot of the dry residue was saponified in a shaking incubator for 60 min at 55°C under N_2 using 1 mL of a 6% KOH solution in MeOH (w/v). After the addition of 1 mL of NaCl solution (10%, w/v), the mixture was placed in the freezer for 15 min. Then, 2.5 mL of hexane: diethyl ether (3:1, v/v) was added, and the mixture was vortexed and centrifuged at 433 x *g* for 3 min (this step was repeated until the aqueous phase was colorless). The organic layers were combined and the solvent was removed under nitrogen stream. The residue was stored at -80 °C under argon atmosphere until UPLC analysis. The dry residue was dissolved in the injection solvent immediately before the analysis (Delpino-Rius et al. 2014).

2.6 UPLC-PDA-APCI-MS/MS Analysis

UPLC assay was carried out using an ACQUITY UPLC binary system. Mass detection was carried out using a Waters XEVO-TQD tandem quadrupole mass spectrometer (Manchester, UK). MassLynxTM software version 4.1 (Waters, Milford, MA) was used to control the instruments, and also for data acquisition and processing. The first condition of UPLC chromatographic separations was performed on a reversed-phase column ACQUITY UPLCr BEH C18 130 Å, 1.7 µm, 2.1 x 150 mm (Waters). Mobile phase consisted of solvent A: acetonitrile (ACN): MeOH 7:3, v/v and solvent B: water 100%. The gradient program used is shown in Table 1. The second condition was used for the **sample 50cyt**, and included a reversed-phase column Symmetry C18 100 x 2,1 mm x 3,5 µm. Mobile phase consisted of solvent A MeOH 100%: and solvent B: water/MeOH 20/80, v/v. The gradient program used is shown in Table 2. For both conditions, the column and sample temperatures were set at 32 and 25˚C, respectively. Injection volume was 5 µL. Optimized MS conditions are listed in Table 3. Each sample extract for LC analysis was dissolved in 1 mL of the injection

solvent [ACN: MeOH 7:3, v/v]: acetone 2:1, v/v. Before use, all solutions were filtered through Millex 0.2 µm nylon membrane syringe filters (Millipore, Bedford, MA).

Table 1. Condition 1: gradient profile used in the separation of carotenoids by UPLC.

Time (min)	Flow rate (mL/min)	A[1] (%, v/v)	B[2] (%, v/v)
Initial	0.5	75	25
1.03	0.5	75	25
9.88	0.5	95.1	4.9
11.38	0.7	100	0
20.18	0.7	100	0
21	0.5	75	25
23	0.5	75	25

[1] A: acetonitrile (ACN): MeOH 7:3; [2] B: water 100%.

Table 2. Condition 2: gradient profile used in the separation of carotenoids by UPLC.

Time (min)	Flow rate (mL/min)	A[1] (%, v/v)	B[2] (%, v/v)
Initial	0.5	20	80
15	0.5	100	0
25	0.7	100	0
26	0.7	20	80
33	0.5	20	80

[1] A: MeOH 100%; [2] B: water/MeOH 20/80, v/v

Table 3. MS conditions.

MS conditions	APCI
Polarity	Positive
Corona (kV)	4.0
Cone (V)	30
Extractor (V)	3
RF (V)	0.1
Source temperature (^{o}C)	150
Probe temperature (^{o}C)	450
Cone gas flow (L/h)	10
Desolvation gas flow (L/h)	150
Collision gas flow (mL/min)	0.15

Semi-preparative medium-pressure liquid chromatography (MPLC) was carried on Combi Flash Rf (Teledyne Isco, USA). The apparatus was equipped with a REDI SEP Rf GOLD C18, 20-40 µm, spherical particles semi-preparative LC column (26 g) with UV detector at 498 and 387 nm. A gradient elution program was used starting with 100%

solvent A (water: methanol 10:90) to 100% solvent B (ethyl acetate: methanol 10:90) in 32 min at flow of 5 mL/min.

2.7 Sensitivity of strains to UV-B and UV-C radiation

The UV-B (peak at 310 nm) and UV-C radiation (peak at 254 nm) resistance test was conducted as described by Casteliani et al. (2014). The test consisted of inoculating 100 µL of cell suspension (10^4 cells.mL^{-1}), obtained according to protocol described by Myers et al. (2013), at the center of Petri dishes containing nutrient agar (NA), prior to radiation exposure. The plates were distributed in a chamber of UV-B radiation which held two fluorescent lamps (EL UV-B -313, Q -Lab, USA) with a cellulose acetate filter (0.1 mm, Malaga, SP) that inhibited UV-C spectrum, installed 40 cm from the base. The incidence of UV-B obtained in the chamber was measured using a spectroradiometer (Ocean Optics Model USB2000 rad +) and calculated as 0.52 $J.M^{-2}.s^{-1}$. The bacteria-containing plates (without lids) were exposed for 0 (control), 30, 60, 90, 120 and 150 min. The UV-C germicide radiation (TUV-30W/G30 Philips) was carried out in a security chamber at a distance of 10 cm from the emitting light source exposed for 0, 30, 60, 90, 120, 240 and 600 seconds. The treatments were performed in triplicate. After exposure, plates with pigmented bacteria were incubated for 96 h at 15 °C in the dark to prevent photoreactivation; controls were incubated at 30 °C for 48 h. After incubation, CFUs were counted. *Escherichia coli* ATCC 11775 was used as negative control and *Bacillus safensis* SG-32 (closely related to *B. pumilus;* Link et al. 2004) was used as positive control due to its high resistance to UVR (Kuhlman et al. 2005). Survival curves were obtained from the mean number of CFUs obtained in triplicate on each plate as a function of the exposure time.

2.8 Statistical Analysis

Statistical analysis of the bacterial UV radiation survival was achieved using the regression model with the variable response given by Negative Binomial Distribution. The parameters estimation was a Bayesian inference by the technique of Integrated nested Laplace approximations. The confidence intervals were 95% probability, that is, the parameter is not significant if the range contains zero.

2.9 Evaluation of photostability by UV spectrometry

Photostability was determined by the comparative analysis of the absorption spectrum of the crude extract of pigments in solutions at 200 µg/mL isopropanol in the Gehaka model UV-380G (São Paulo, Brazil) in the range of 250 to 700 nm. Samples were subjected to UV-A visible irradiance (UVA: 86.1%: and visible light: 13.9%) close to 8 mW/cm^2 emitted by a Philips UV-A Actinic BL/10 lamp (Eindhoven Netherlands) for 52 minutes, with a total dose of 27.5 J/cm^2 (Whitehead and Hedges 2005; Freitas et al. 2015) compared to those which were left out of the light. The ratio of the integrating area under the curve of the irradiated spectrum by the integrating area of the non-irradiated absorption spectrum yielded the determination of photostability in the range of UV-B (280-320 nm), UV-A (320-400 nm) and visible (400-700 nm), considering effects after irradiation under UVA lamp.

2.10 Phototoxicity test in cell culture (3T3 NRU)

The 3T3 Neutral Red Uptake Phototoxicity Test was performed according to INVITTOX Protocol No. 78 (Spielmann et al. 1998; Ecvam Db-Alm 2008), and Organisation for Economic Cooperation and Development Test Guideline nº432 (OECD TG 432) (OECD 2004), using 3T3 Balb/c fibroblasts (L1, ECACC No. 86052701). For this purpose, after evaluation of fibroblasts sensibility to the UV-A radiation, two 96-well plates were used for the pigment test, one to determine the cytotoxicity (absence of radiation, -UV-A plate) and another for phototoxicity (presence of radiation, +UV-A plate). For that, firstly 100 µL of a cell suspension of 3T3 fibroblasts in Dulbecco's Modification of Eagle's Medium (DMEM) containing New Born Calf Serum and antibiotics (1×10^5 cells/mL, 1×10^4 cells/well) was dispensed in two 96-well plates. After a 24 h period of incubation (7.5% CO_2, 37 °C), plates were washed with 150 µL of Dulbecco's Phosphate Buffered Saline (DPBS) and 8 different concentrations 6.81 µg/mL, 10 µg/mL, 14.7 µg/mL, 21.5 µg/mL, 31.6 µg/mL, 46.4 µg/mL, 68.1 µg/mL and 100 µg/mL of the test chemicals or combination were applied in sextuplicate in the 96-well plates. After 1 h incubation, the +UV-A plate was irradiated for approximately 20 min with 7 mW/cm^2 achieving 9 J/cm^2 of UV-A radiation from UV-sun simulator, type SOL-500 (Dr. Hönle, Germany). In the meantime, the UV-A plate was kept in a dark box. Culture medium replaced the test solutions and the plates were incubated

overnight. Neutral Red medium was added in each well and after 3 h of incubation period, cells were washed with DPBS and a desorbent (ethanol/acetic acid) solution was added. Neutral red extracted from viable cells formed a homogeneous solution and the +UV and −UV plates were analyzed in a microplate reader at 540 nm.

The Phototox Version 2.0 software (obtained from ZEBET, Germany) was employed for the concentration-response analysis (IC50). The mean photoeffect (MPE) is a statistical comparison of the dose-response curves obtained with and without UV (Holzhütter 1997)(Holzhütter 1997)(Holzhütter 1997)(Holzhütter 1997)(Holzhütter 1997). According to the OECD TG nº 432, a test substance with MPE values below 0.1 is predicted to be “non-phototoxic”, values between 0.1 and 0.15 are predicted to be ''probably phototoxic'' and values greater than 0.15 are predicted to be ''phototoxic'' (OECD 2004; Kejlová et al. 2007). Results are the mean of at least two independent experiments (± SEM). Norfloxacin and *L*-histidine were used as positive and negative controls, respectively.

2.11 Antioxidant activity

2.11.1 Scavenger assay of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH)

Free radical-scavenging activity was measured using a method adapted by Brand-Williams et al. (1995). The experiment was performed on freshly prepared ethanolic solutions of DPPH (0,004% w/v). In brief, 50 µL of extract in different concentrations was mixed with 250 µL of DPPH solution in a transparent microplate with 96 wells (Costar, Cambrige, Ma). After 30 min of reaction, the absorbance of the remaining DPPH was measured at 517 nm on a microplate reader NOVOstar (BMG Labtech®, Offenburg, Germany) at 25 °C in a dark chamber. Antioxidant activity was expressed as a percentage of the absorbance of the control DPPH solution, obtained from the following equation: % Activity = $[(A_{DPPH}-A_{sample})/A_{DPPH})] \times 100$, where A_{DPPH} is the absorbance value of the control, and A_{sample} is the absorbance value of the test solution. Trolox was used as a standard using a calibration curve (15 - 250 µM), and results were expressed as µmol Trolox equivalent (TE)/mg of pigment. These results were obtained in triplicate and expressed as mean ± SD.

2.11.2 Scavenger of the 2,2-azino-bis (3-ethylbenzothiazoline) -6-sulphonic acid ($ABTS^{+}$)$^{-}$

The $ABTS^{\bullet+}$ scavenging capacity assay was determined as described by Le et al. (2007). The method is based on the decolorization of the ABTS radical cation to determine the antioxidant potential of samples. The solution of ABTS radical cation was prepared in advance by reacting aqueous ABTS solution (7 mM) with potassium persulfate (2.45 mM). In the analysis, diluted $ABTS^{\bullet+}$ solution with an absorbance of 0.70 ± 0.02 at 734 nm was employed. The assay was performed using quartz cuvette with black mask and the reaction system was composed of 200 µL of sample and 1000 µL of $ABTS^{\bullet+}$solution, followed by 6 min incubation at room temperature. The absorbance values were measured on a spectrophotometer (DU 640, Beckman, Coulter Inc., CA, USA) at 734 nm in triplicate. Free radical scavenging activity was expressed as a percentage of the absorbance of the control $ABTS^{\bullet+}$, obtained with the following equation: % Activity = $[(A_{ABTS\bullet+} - A_{sample})/A_{ABTS\bullet+}] \times 100$, where $A_{ABTS\bullet+}$ is the absorbance value of the $ABTS^{\bullet}$ control, and A_{sample} is the absorbance value of the extract solution. A calibration curve was plotted for absorbance reduction and concentration of the Trolox (10 – 250 µM) and the results were expressed as µmol Trolox equivalent (TE)/g of pigment. These results were obtained in triplicate and expressed as mean ± SD.

2.11.3 Oxygen radical absorbance capacity ($ORAC_{FL}$) assay

The $ORAC_{FL}$ assay was described by Prior et al. (2003) and modified by Dávalos et al. (2004). Reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 200 µL. Antioxidant sample (20 µL) and fluorescein (120 µL; 70 µM in final concentration) solutions were mixed in one of the 96 wells of a black microplate (Costar, Cambridge, Ma). Then, 60 µL of an AAPH solution (final concentration 12 mM) were added and fluorescence was checked every cycle of 60 s for 80 cycles. The automated ORAC assay was performed on a NovoStar Microplate reader (BMG Labtech, Offenburg, Germany) with fluorescence filters (excitation, λ 485 nm; emission λ 520 nm). The experiment was conducted at 37^{o}C and pH 7.4 with a blank sample in parallel. The result was calculated using the differences of areas under fluorescein (FL) decay curves (net AUC) between the blank and sample and was

expressed as equivalent for µmol of Trolox (µmol/TE/g) per µmol of pigment, as described in the following equation: AUC1 + $\Sigma f_i / f_{0,}$ where f_0 is the initial fluorescence (t=0) and f_i is the fluorescence obtained at t=i (min). Net AUC = $AUC_{sample} - AUC_{blank}$

Net AUC was plotted against sample concentration and results were compared to the standard curve (Net AUC *versus* Trolox concentration). The equivalence of Trolox was given by the angular coefficient of Trolox curve concentration (µM) *versus* sample concentration (µM). All assay was performed in three independent replicates.

3. RESULTS

3.1 Carotenoid Identification

Identification of carotenoids is described below according to the bacterial strain producing pigment and the retention time (RT) in the C18 column. Characteristics of the carotenoids separated in the UPLC/MS system are summarized in Table 4. The UV, MS and MS/MS spectra are presented in Electronic Supplementary Material (ESM).

The PDA/UPLC chromatogram of pigment from *A. agilis* 50cyt is presented in ESM 0 and 1.

Bacterioruberin diglucoside (peak at the RT 4.99 min) and *cis-bacterioruberin diglucoside* (peak at the RT 5.55 min) (ESM 1, 2 and 3): their mass spectra allowed the identification of both isomers with the protonated molecule at *m*/*z* 1065 and fragments at *m*/*z* 1048 $[M+H-18]^+$ (-18 means a loss of a water molecule) and 1030 $[M+H-18-18]^+$.

Bacterioruberin monoglucoside (peak at the RT 5.64 min) and *cis-bacterioruberin monoglucoside* peak at the RT 6.18 min (ESM 4 and 5): their mass spectra allowed the identification of both isomers with the protonated molecule at *m*/*z* 903 and fragments at *m*/*z* 885 $[M+H-18]^+$ and 867 $[M+H-18-18]^+$.

All-trans bacterioruberin peak at the RT 6.34 min (ESM 6): the UV-visible spectrum, *λ*max at 467, 494, and 528 nm, and *cis-bacterioruberin* (peak at the RT 6.77 min) (ESM 7): their mass spectra allowed the identification of both isomers with the protonated molecule at *m*/*z* 741 and fragments at *m*/*z* 723 $[M+H-18]^+$ and 705 $[M+H-18-18]^+$. The protonated molecules were similar to the data from literature for bacterioruberin

variations from *A. agilis* and *Halobacterium* species (Fong et al. 2001; Britton et al. 2004).

The PDA/UPLC chromatogram of pigment from *A. psychrochitiniphilus* is presented in ESM 0 and 8.

Decaprenoxanthin isomers (peaks at the RT 11.61, 11.85 and 12.09 min) (ESM 9) at the UV-visible spectrum, *λ*max at 416, 440, and 468 nm, were very similar. Their mass spectra allowed the identification of the three isomers with the protonated molecule at *m*/*z 705* and fragments at *m*/*z* 689 $[M+H-16]^+$, 669 $[M+H-18-18]^+$, 595 $[M+H-18-92]^+$. The *cis-decaprenoxanthin* (peaks at the RT 12.35 and 12.51 min) (ESM 11) showed characteristic UV-visible spectra. Their mass spectra resulted in the identification of both *cis*-isomers with the protonated ion at *m*/*z* 705 and fragments at *m*/*z* 787 $[M+H-18]^+$ and 669 $[M+H-18-18]^+$. The protonated ion at *m*/*z* 705 was similar to the data from literature for decaprenoxanthin isolated from other *Arthrobacter* species (Arpin et al. 1975; Britton et al. 2004; Giuffrida et al. 2016)

The PDA/UPLC chromatograms of pigment from *Z. laminarie* 465 are presented in Figs. ESM 0 and 12.

Zeaxanthin (peak at the RT 7.93 min) (ESM 13) was identified as *all-trans*-zeaxanthin considering the UV-visible spectrum, *λ*max at 453 and 479 nm, and confirmed by coelution with the *all-trans*-zeaxanthin standard (ESM 14). As expected, the mass spectrum showed the protonated molecule at *m*/*z* 569 and fragments at *m*/*z* 551 $[M+H-18]^+$.

Three carotenes (peaks at the RT 10.33, 10.88 and 11.8 min) from *Z. laminarie* 465 remain unidentified (ESM 15, 16 and 17)

β–cryptoxanthin (peak at the RT 12.17 min) (ESM 18): Identified as *all-trans*- β – *cryptoxanthin*, with the UV-visible spectrum similar to those from zeaxanthin with maximum absorbance at 452 nm. The protonated molecule was detected at *m*/*z* 553, along with less intense fragments at *m*/*z* 535 $[M+H-18]^+$ and 461 $[M+H-92]^+$. The identification was confirmed through coelution with the *all-trans*- β -cryptoxanthin standard.

β –carotene (peak at the RT 15.33 min) (ESM 19): Identified as *all-trans*- β – carotene, with the UV-visible spectrum similar to those from zeaxanthin with maximum absorbance at 451 nm. The protonated molecule was detected at *m*/*z* 538, along with less intense fragments at *m*/*z* 444 $[M+H-92]^+$. The identification was confirmed through coelution with the *all-trans*- β – carotene standard.

Phytoene (peak at the RT 16.69 min) (ESM 20) was identified as phytoene by comparing the UV-visible spectrum (*λ*max and fine structure) with that given in the literature (Britton et al. 2004). The mass spectrum showed the protonated molecular at *m*/*z* 545. It was only detected in *Z. laminarie* 465 sample.

Table 4. Summary of the results obtained by UPLC/PDA/MS.

Sample	Retention Time (min)[a]	Carotenoid	λ_{max} (nm)[b]	%III/II[c]	$[M+H]^+$ (*m/z*)	fragment ions (*m/z*)
Arthrobacter agilis 50cyt	4.99	bacterioruberin diglucoside	468, 495,529	56	1065	1048[M+H-18], 1030 [M+H-18-18]
	5.55	*cis*-bacterioruberin diglucoside	370,386 - 460, 489,522	44	1066	1048[M+H-18], 1030[M+H-18-18]
	5.64	bacterioruberin monoglucoside	465, 494,528	58	903	885[M+H-18], 867[M+H-18-18]
	6.18	*cis*-bacterioruberin monoglucoside	369,386 - 462, 490,521	58	903	885[M+H-18], 867[M+H-18-18]
	6.34	*all-trans*-bacterioruberin	467,494,528	63	741	723[M+H-18], 705[M+H-18-18]
	6.77	*cis*-bacterioruberin	369,386 - 460, 487, 521	42	741	723[M+H-18], 705[M+H-18-18]
Arthrobacter psychrochitiniphilus 366	11.61	Decaprenoxanthin	416, 440, 468	95	705	689[M+H-16], 669[M+H-18-18], 595 [M+H-18-92]
	11.85	Decaprenoxanthin	417, 441, 467	97	705	689[M+H-16], 669[M+H-18-18], 595 [M+H-18-92]
	12.09	Decaprenoxanthin	417, 441, 469	94	705	689[M+H-16], 669[M+H-18-18], 595 [M+H-18-92]
	12.35	*cis*-decaprenoxanthin	314,328 - 412, 435, 463	65	705	687 [M+H-18], 669[M+H-18-18] 391
	12.51	*cis*-decaprenoxanthin	314,328 - 412, 435, 464	71	705	687[M+H-18], 669[M+H-18-18] 663

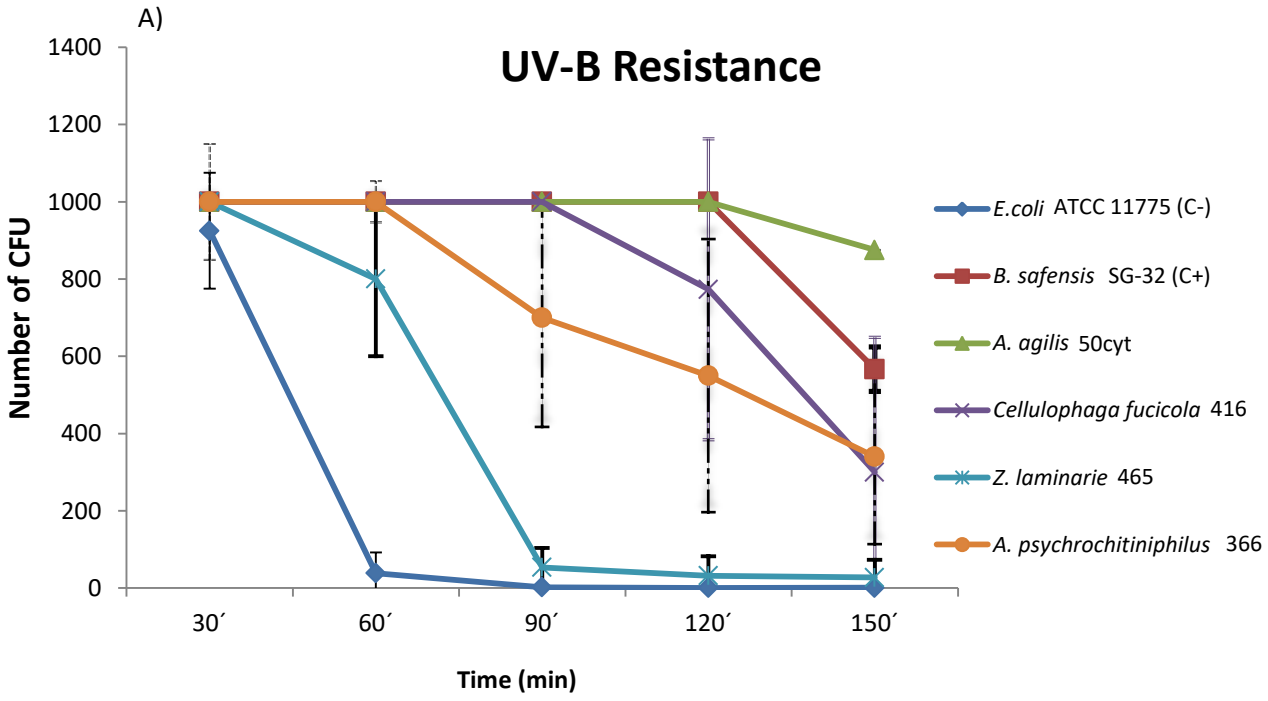
Zobellia laminarie 465	7.96	Zeaxanthin	453, 481	30	569	551[M+H-18]
	10.33	Unidentified	339 - 447	0	639	569, 551
	10.88	Unidentified	360 - 469,501	55	715	705
	11.8	Unidentified	418, 440, 469	113	657	627
	12.17	β -cryptoxanthin	452	20	553	535[M+H-18]
	15.32	β -carotene	451	7	537	-
	16.69	Phytoene	286	15	545	-

[a] Retention time on the C18 column. [b] Linear gradient of ACN:MeOH/water. [c] Defined spectral fine structure

3.2 UV-B and UV-C resistance

When exposed to UV-B radiation, the most successful strains were *A. agilis* 50cyt (around 78% survival after 150 min of exposure), followed by the positive control *B. safensis* SG-32 (60%), *Cellulophaga fucicola* 416 (40%), *A. psychrochitiniphilus* 366 (35%) and *Z. laminariae* 465 (10%) (Fig. 2A). The *E. coli* survived only 60 min of exposure. Statistical analyses revealed that all bacteria were significantly different from the negative control *E. coli* for UV-B radiation and *A. agilis* 50cyt had similar survival rates as *B. safensis* SG-32.

The UV-C resistance test indicated that *A. agilis* 50cyt has a statistically longer survival rate than *E.coli*, but it showed to be more sensitive than *B. safensis* SG-32. *Z. laminariae* 465 and *A. psychrochitiniphilus* 366 did not present UV-C resistance (Fig. 2B).



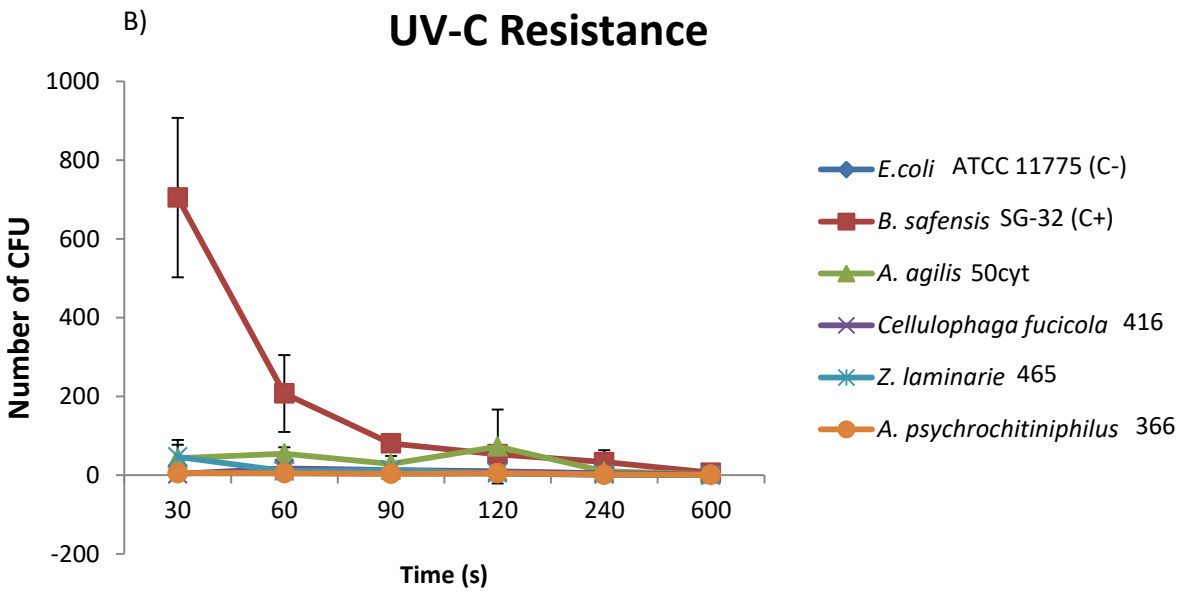


Fig 2. (A) UV-B resistance and (B) UV-C resistance. Suspensions of 10^3 cells were irradiated with UV-B (310 nm) and UV-C (254 nm) respectively. The surviving fraction CFU was determined as a function of time. *E. coli* was included as negative control and *B. safensis* as positive control.

3.3 Photostability and phototoxicity tests

A pigment to be considered photostable should not decrease more than 20% during the exposure test (Gaspar and Maia Campos 2006). Thus, the pigment crude extract from *Z. laminarie* 465 was considered photostable (92.3%) under UV-B and stable under UV-A (80.1%), but less stable under the visible light (74.2%) (Fig. 3). The pigment from *A. agilis* 50cyt was photostable under UV-B (92.7%) but not stable under UV-A and visible spectra. The decaprenoxanthin pigment from *A. psychrochitiniphilus* 366 was considered completely photostable under UV-B (113.5%) and photostable under UV-A (90.3%) and visible spectra (91.3%).

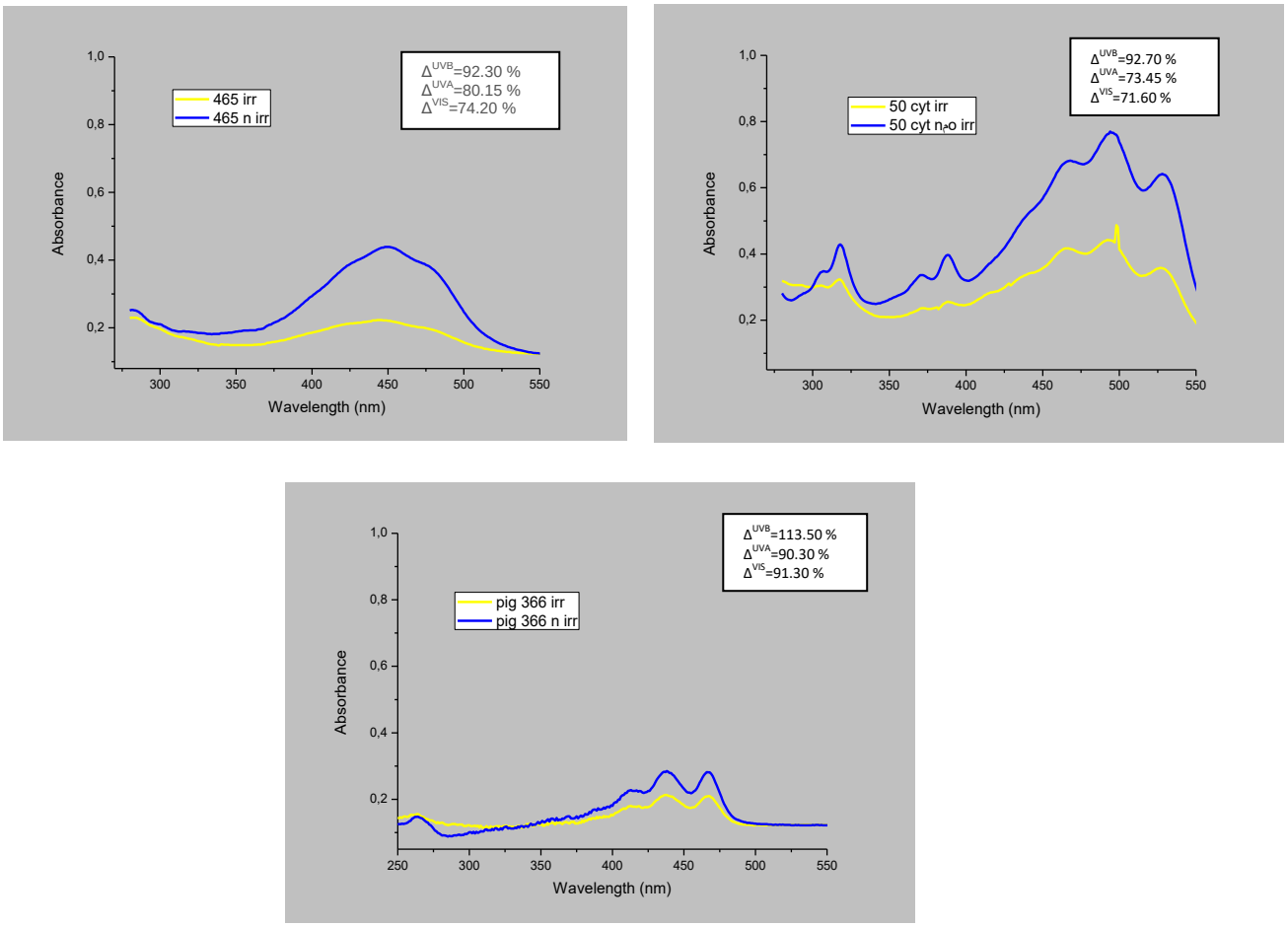


Fig 3. Photostability of pigments (%) and spectral behavior when exposed (yellow line) or not (blue line) to UVA irradiance .

3.3.1. Phototoxicity test in cell culture (3T3 NRU)

The pigments purified in the semi-preparative MPLC resulted in three fractions of the *Z. laminarie* 465 named 35-40, 41-44 and 56-59 and three fractions for *A. agilis* 50cyt named 18-21, 23-24 and 28-29. The pigment of *A. psychrochitiniphilus* 366 was not performed. These purified samples were used in the phototoxicity tests.

According to the results obtained in the 3T3 NRU test in fibroblasts from mice (Table 5, ESM 21 and 22) the only pigment that did not show phototoxic potential was fraction 28-29 of the pigment from *A. agilis* 50Cyt. In both tests performed in duplicate, the MPE was lower than 0.1, and IC50+UV was higher than 60 µg/ mL, which correspond to criteria of non-phototoxicity.

Table 5. Results of 3T3 NRU phototoxicity test.

Sample	Phototoxicity	MPE[b]	IC50-UV[c] (µg/ mL)	IC50+UV[c] (µg/ mL)
Norfloxacin (C+)[a]	Phototoxic	0.615/0.909	-	4.2/6.0 and 26.2
465 fraction 35-40	Phototoxic	0.559/ 0.676	-	15.4/23.18
465 fraction 41-44	Phototoxic	0.145/ 0.285	-	55.7/48.4
465 fraction 56-59	Phototoxic	0.595/ 0.341	-	11.7/43.47
50cyt fraction 18-21 (bacterioruberin diglucoside)	Cytotoxic	0.127/0.107	69.2/44.8	26.47/20.44
50cyt fraction 23-24 (bacterioruberin monoglucoside)	Phototoxic	0.931/0.909	-	0.76/2.65
50cyt fraction 28-29 (*all-trans*-bacterioruberin)	Non phototoxic	0.088/ -0.034	-	61.59/74.30

[a] Norfloxacin used as positive control. [b] Mean photoeffect - MPE < 0.1 non-phototoxic; MPE>0.1 and <0.15: probably phototoxic; MPE>0.15: phototoxic. [c] IC50 concentration that kills 50% of cells, the lower this value, the more toxic the substance is.

3.4 Antioxidant capacity

The crude extract of pigment from *A. agilis* 50cyt, *A. psychrochitiniphilus* 366 and *Z. laminarie* 465 were tested in different antioxidant assays, the results obtained are expressed in µmol Trolox equivalent (TE)/mg of pigment in Table 6. In order to compare with other data from literature, one should multiple 1,000 fold to achieve µmol Trolox equivalent (TE)/grams (g) of pigment, resulting for example in 5,950 µmol Trolox equivalent (TE)/g of pigment in DPPH test for 50cyt.

The pigment crude extract from *A. agilis* 50cyt presented the highest antioxidant activities in DPPH (5.95), ABTS-H (5.31) and ORAC-H (2.24). The crude pigment from *A. psychrochitiniphilus* 366 presented the highest values for ORAC-L (9.92) and ORAC-CAT (11.95), and the pigment from *Z. laminarie* 465 presented the highest values for ABTS-L (7.25).

Table 6. Antioxidant capacity of pigments from Antarctic bacteria.

	DPPH[c]	ABTS-H[d]	ABTS-L[e]	ORAC-H[f]	ORAC-L[g]	ORAC-CAT[h]
Sample	µmol Trolox equivalent (TE)/mg of pigment					
50 Cyt	5.95 ± 0.20 (I% = 20.54)	5.31 ± 0.11 (I%= 27.44)	5.32 ± 0.09 (I% = 26.57)	2.24 ± 0.62	7.34 ± 1.09	9.58
366	3.24 ± 0.02 (I% = 11.00)	3.38 ± 0.47 (I%= 17.09)	2.02 ± 0.23 (I% = 10.87)	2.03 ± 0.09	9.92 ± 0.51	11.95
465	4.59 ± 0.52 (I% = 15.77)	4.37 ± 0.65 (I%= 23.73)	7.25 ± 0.59 (I% = 34.32)	1.18 ± 0.10	8.03 ± 1.42	9.21

[a] Values expressed as the mean of triplicate ± standard deviation. [b] Percent inhibition % I. [c] **DPPH**, Sequestration of the 2,2-diphenyl-1-picrylhydrazyl radical. [d] **ABTS-H**, Capture of the 2,2-azino-bis (3-ethylbenzothiazoline) -6-sulphonic acid (ABTS +) radical of the hydrophilic fraction. [e] **ABTS-L**, Capture of the 2,2-azino-bis (3-ethylbenzothiazoline) -6-sulphonic acid (ABTS +) radical of the lipophilic fraction. [f] **ORAC-H**, Absorption capacity of the oxygen radical from hydrophilic fraction. [g] **ORAC-L**, Absorption capacity of the oxygen radical from lipophilic fraction. [h] **ORAC-CAT**, Absorption capacity of the total oxygen radical.

4. DISCUSSION

Pigments are widely found in bacteria. Among them, the most abundant groups are carotenoids. They are produced by a wide variety of organisms, from non-photosynthetic prokaryotes to higher plants, with more than 700 different structures identified to date. They occur in yellow, orange or red colors. Biosynthesis of their carbon skeleton is based on the condensation of isoprene units, conferring a variety of structures through modifications of the carbon chain (Schwender et al. 1996). Because of their color and biological activity (antioxidant action), carotenoids are used commercially as food colorings, feed supplements and, more recently, for cosmetic and pharmaceutical purposes (Britton 1995; Britton et al. 2004). As an alternative to synthetic pigments, bacterial pigments are promising because of their likely higher biodegradability and compatibility with the environment, and still offer great potential for several other applications (Venil et al. 2013).

The three minimum recommended procedures for identification and structure elucidation of carotenoids were fulfilled in this work: co-chromatography with an authentic standard (except for bacterioruberin, decaprenoxanthin and phytoene), UV/Vis spectrum, and mass spectrum of a quality that allows identification of the molecular ion (Britton et al. 2004; Takaichi 2014). Herein the significant carotenoids identified from *A. agilis* 50 were: *all-trans*-bacterioruberin, bacterioruberin monoglucoside and diglucoside; from *A. psychrochitiniphilus 366* the decaprenoxanthin, and from *Z. laminarie* 465 the zeaxanthin, β –cryptoxanthin, β –carotene and phytoene.

Bacterioruberin is a C50 carotenoid (ESM 23) and has already been described as an effective oxidation protector, with protective effects against UV-radiation and also against oxidative DNA-damaging agents (Saito et al. 1997; Shahmohammadi et al. 1997; Shahmohammadi et al. 1998; Kottemann et al. 2005). Nevertheless, this is the first report on the description of different fractions of bacterioruberin with different behaviors related to the phototoxic potential evaluated in the *in vitro* method 3T3 NRU PT. The fractions identified as bacterioruberin mono and diglucoside presented a high level of phototoxicity and cytotoxicity against fibroblasts while the *all-trans*-bacterioruberin did **not** present any phototoxic activity, opening perspectives for its future use as UV-filter. The differences observed in phototoxicity of all fractions of bacterioruberin might be explained by the different solubility (due to monoglucoside and diglucoside) of the molecules, which may lead to discrepancies in terms of their bioavailability in the assay. Since 2000, the *in vitro* 3T3 NRU phototoxicity test is the only *in vitro* test required for evaluating the acute phototoxicity of a chemical (Augustin et al. 1997). In other words, when a substance does not present a phototoxic potential, other *in vitro* studies such as permeation or bioavailability or *in vivo* assays are not required to confirm photosafety results, since the 3T3 NRU PT test is very sensitive and does not show false negative results (Spielmann et al. 1998; Ceridono et al. 2012). However, this monolayer culture of fibroblasts is a simple basic system as compared to the three-dimensional architecture of skin involving connections and interactions between different cells types and extra cellular matrix. The molecular

weight of these carotenoids is higher than 500 Da, indicating that they would have low skin penetration in the outer layers of the human skin, resulting in the lack of *in vivo* phototoxicity (Maciel et al. 2018). For this reason, further studies of efficacy of the pigment as a SPF should be conducted using skin models to prove the use of this compound as an UV-filter or as an adjuvant for cosmetics formulation.

The Prokaryotic Carotenoid Database (ProCarDB) (Nupur et al. 2016) contains a list of carotenoids that is supposed to be detected in *Zobellia galactanivorans*, a bacterium closely related to *Zobellia laminarie*, based on genome scan. Among the genes that were retrieved from this bacterium are *CrtI*, *CrtY*, *CrtZ*, and *CrtA* that encode for phytoene, β – carotene, zeaxanthin and a C50 carotenoid, respectively. This is the first time that carotenoid pigments from *Z. laminarie* are detected, isolated and characterized. Phytoene is a colorless carotene which is the biosynthetic precursor of carotenoids (Britton 1995). It is produced from two molecules of geranylgeranyl pyrophosphate C20 (GGPP) by the action of the enzyme phytoene synthase to build the C40 carotenoid. β –carotene is a C40 carotene, and zeaxanthin and β – cryptoxanthin are C40 xanthophylls (ESM 24). These pigments are widespread amongst microorganisms (Hertzberg and Jensen 1966; Kushwaha et al. 1972; Kleinig et al. 1977; Takaichi et al. 1990; Yokoyama et al. 1996; Sajilata et al. 2008). In prokaryotes, carotenoids are located mainly in membranes, due to its lipophilicity, what makes them useful photo-protectants and antioxidant agents (Gammone et al. 2015), ensuring protection against photodynamic killing (Mathews and Sistrom 1959; Mathews-Roth 1987; Carbonneau et al. 1989; Sajilata et al. 2008). The combined extract containing those carotenoids resulted in high antioxidant activity of *Z. laminarie* 465. Due to the possible interference of pigments with the determinations in the DPPH colorimetric method (Correa-Llantén et al. 2012), and the controversial use of ORAC test for carotenoids (Granato et al. 2018), another approach – the ABTS - was employed. The mixture of carotenoids studied in this work showed antioxidant activities higher than typical food antioxidant agents (Ghiselli et al. 1998; Wright et al. 1999; Hartwig et al. 2012). Although the carotenoid extract from *Z. laminarie* 465

showed photostability under UV-A and UV-B, it was not stable under the visible spectrum and was considered phototoxic for further use as sunscreen ingredients.

Decaprenoxanthin from *A. psychrochitiniphilus* is a C50 carotenoid (ESM 25) and occurs in a restricted number of bacterial species (Heider et al. 2014; Giuffrida et al. 2016). This carotenoid presents strong antioxidant properties due to the multiple conjugated double bonds and the hydroxyl group (Cockell and Knowland 1999). Moreover, considering that the UV-A is the main responsible for photochemical reactions in human skin (Freitas and Gaspar 2016), decaprenoxanthin and isomers showed a great potential source of chromophores to be used as UV-A and UV-B filters in sunscreens, as they were photostable under the three different spectra tested. However, phototoxicity tests have not been yet performed.

Thus, despite the commercial sunscreens being effective, there is an increasing concern for searching alternative natural UV-protective pigments. Bacterial carotenoids represent an opportunity for exploring pigments from natural origins which show lower toxicity, no residues, no environmental risks and are readily decomposable (Mohana et al. 2013)**.** For example, 3,3´-dihydroxyisorenieratene and isorenieratene are aromatic carotenoids produced by *Brevibacterium linens* (Rattray and Fox 1999; Wagener et al. 2012) which are efficient photoprotective agents, absorbing UV light. The *all-trans*-bacterioruberin can represent a potential natural product for use as sunscreens, as well as possible use in feed supplement, the same way astaxanthin is used nowadays for salmon feed; however its use as feed or food supplement has not been performed yet.

Finally, the identification of pigments produced by bacteria from Antarctica may help us understand how bacteria thrive in this high UV and cold environments, and open perspectives for further biotechnological application towards a more sustainable and environmental-friendly way of pigments exploitation.

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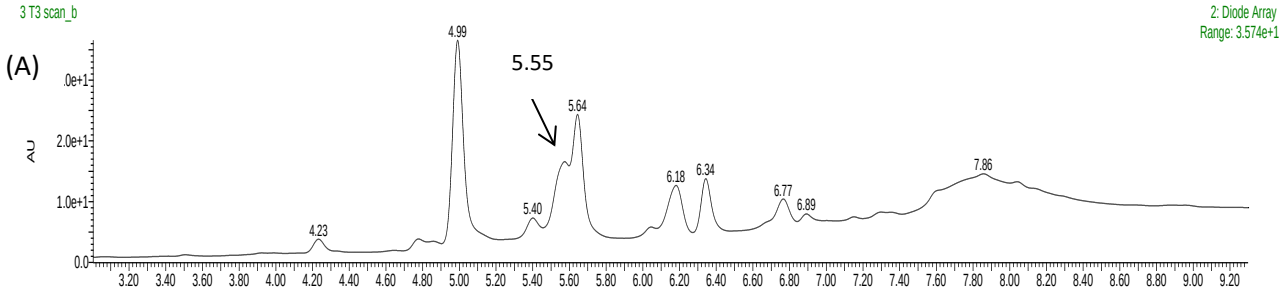
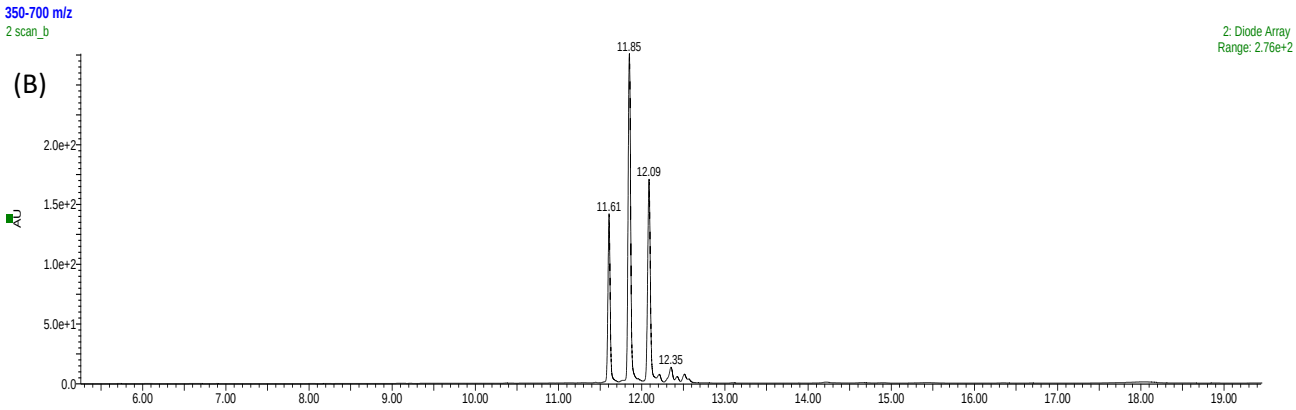
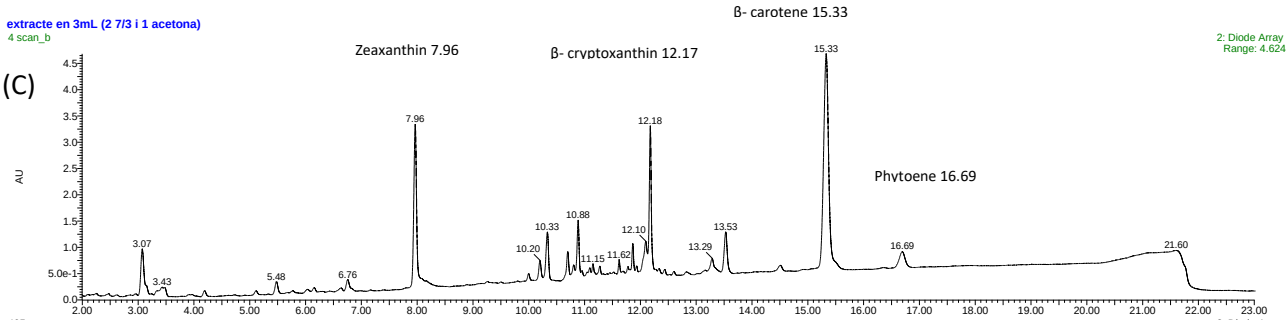
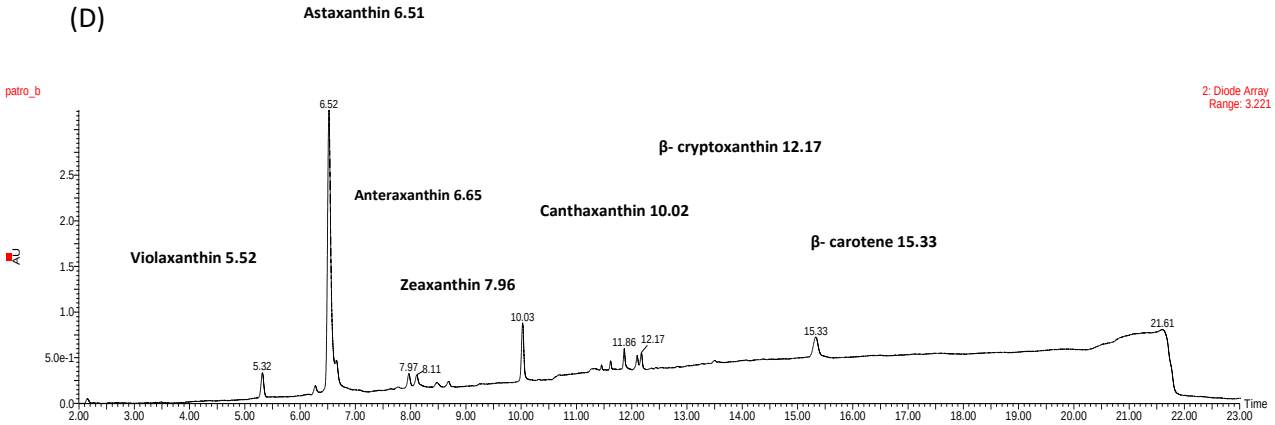
Supplementary Material (ESM)

Chemical characterization and biotechnological applicability of pigments isolated from Antarctic bacteria



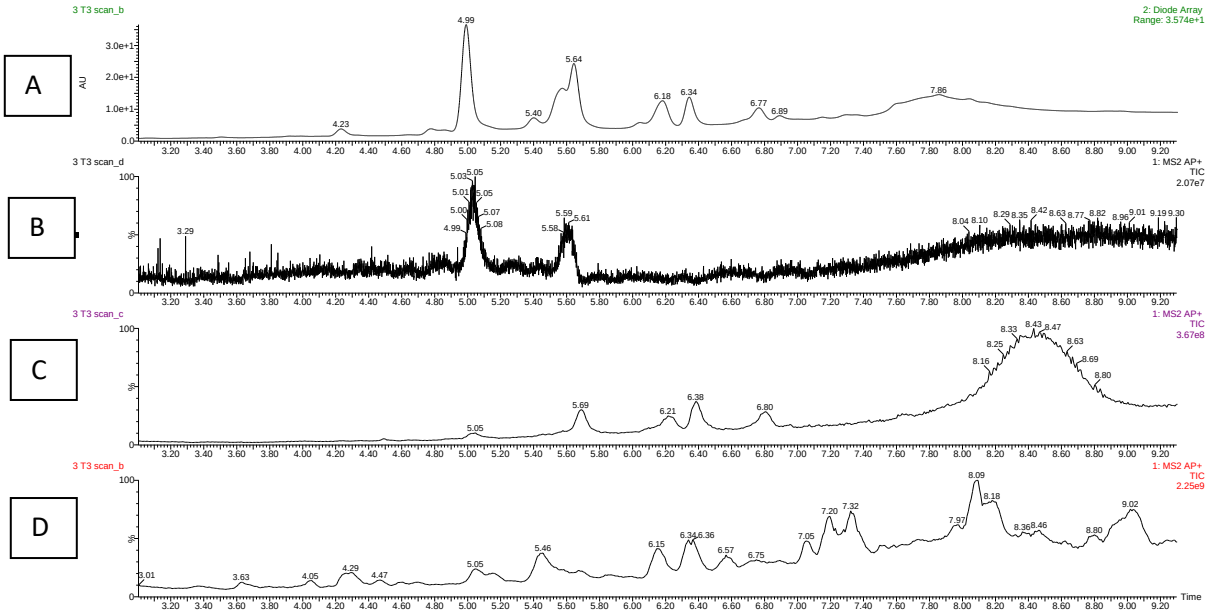




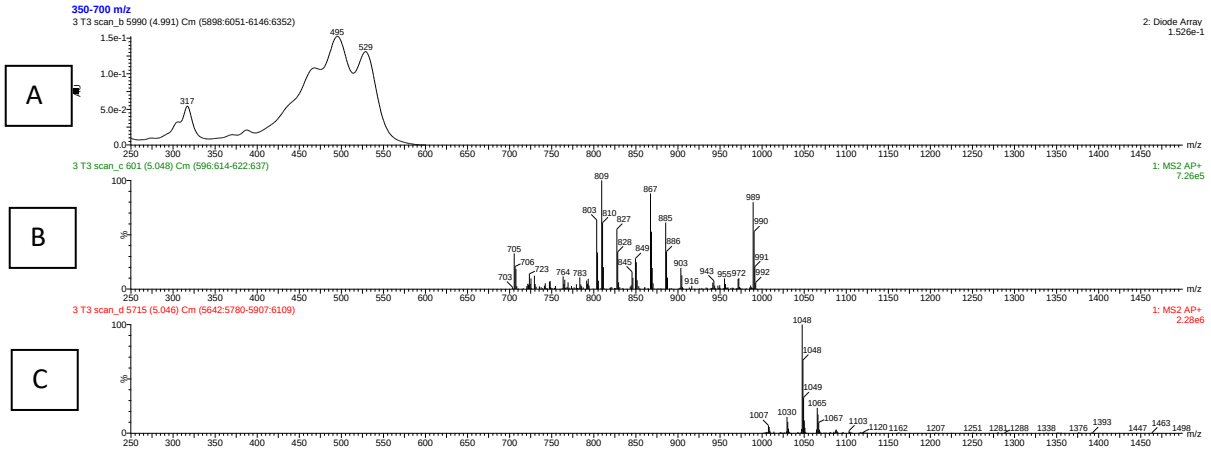


ESM 0. Chromatogram UPLC/PDA from *(A)* saponified extract of *A. agilis* 50Cyt, (B) saponified extract of *A. psychrochitiniphilus* 366, (C) saponified extract of *Z. laminarie* 465 and (D) standards.

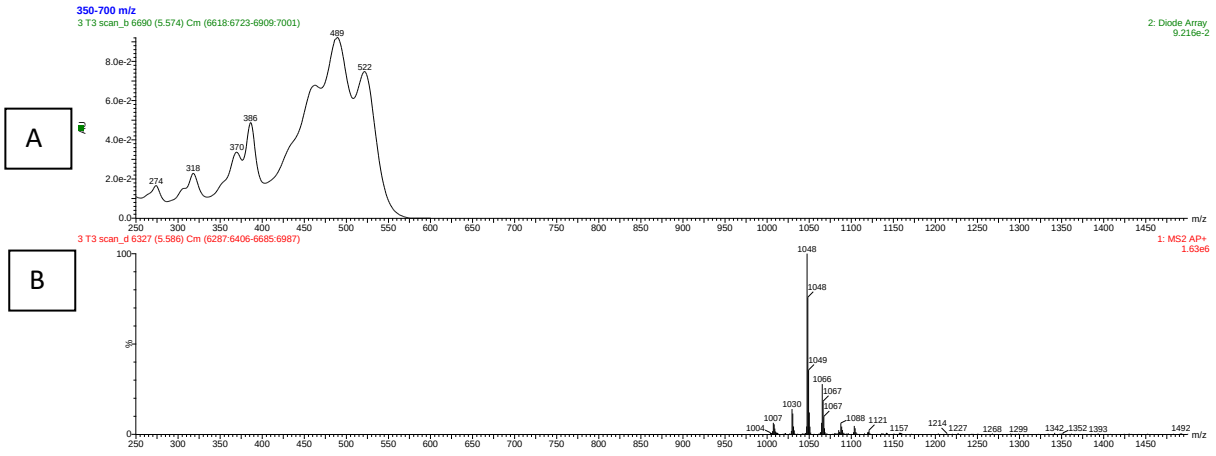
1- *Arthrobacter agilis* 50



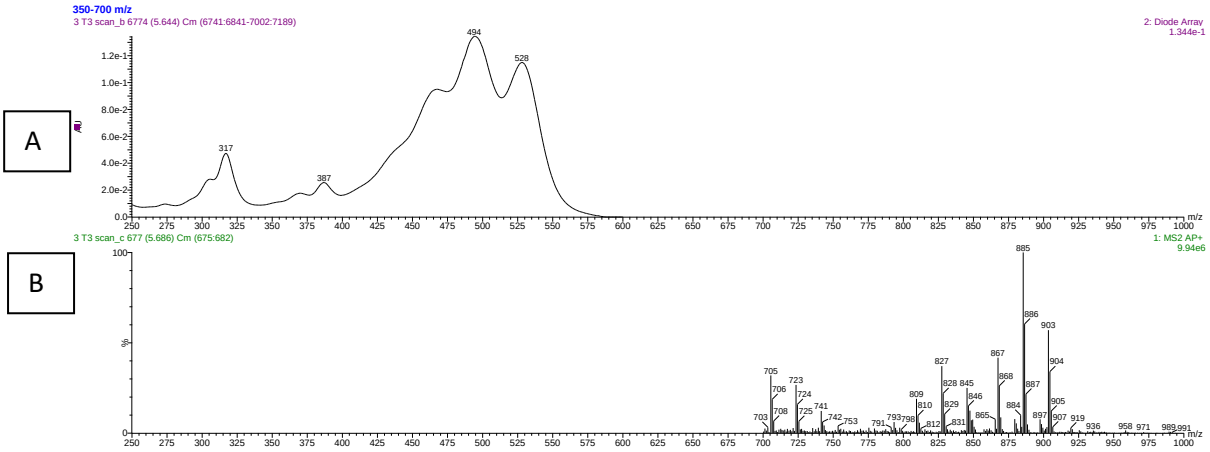
ESM 1. Sample 50: A -UPLC/DAD chromatogram, (B)mass spectrometry (MS) chromatogram 1000 – 1500 *m/z*, (C) 700- 1000 *m/z* and (D)350- 700 *m/z*.



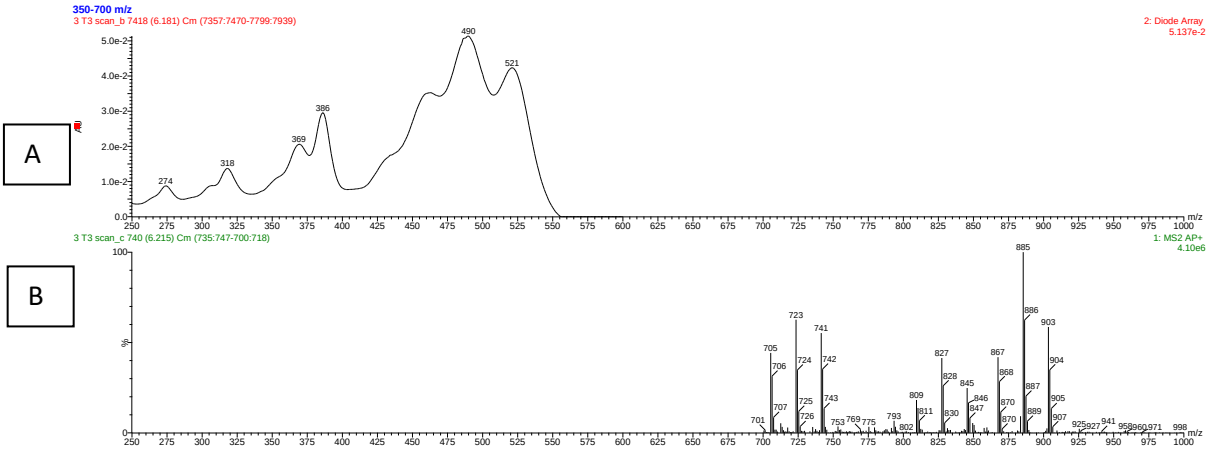
ESM 2. Peak 4.99 – (A) UV/vis, (B) MS 700-1000 *m/z* and (C) MS 1000-1500 *m/z* spectra. The protonated molecule at 1.065 correspondent to bacterioruberin diglucoside.



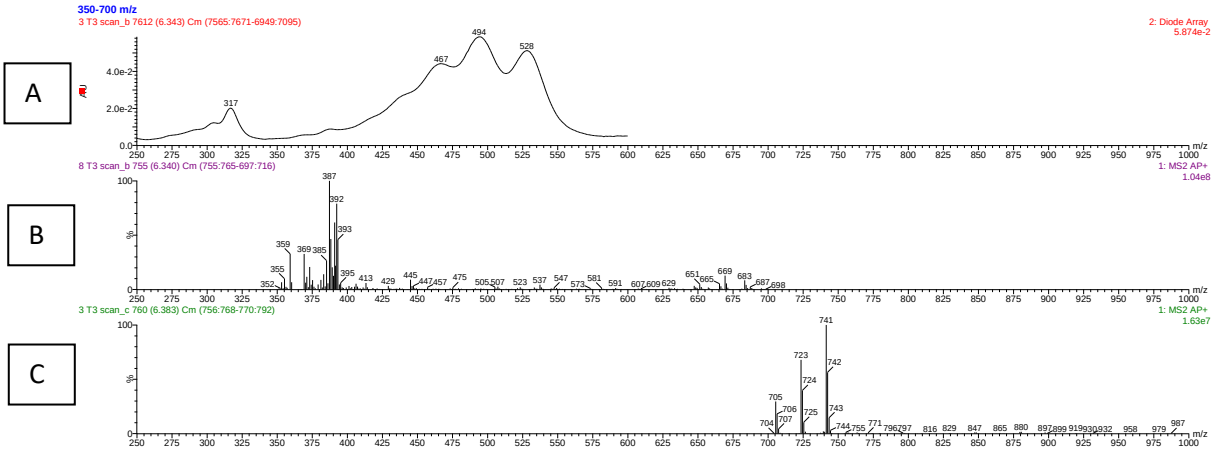
ESM 3. Peak 5.55 (A) UV/vis and (B) MS 1000-1500 *m/z* spectra. The protonated molecule at 1.065 correspondent to bacterioruberin diglucoside, and the *cis peak* at 370 and 386 nm in the UV spectrum.



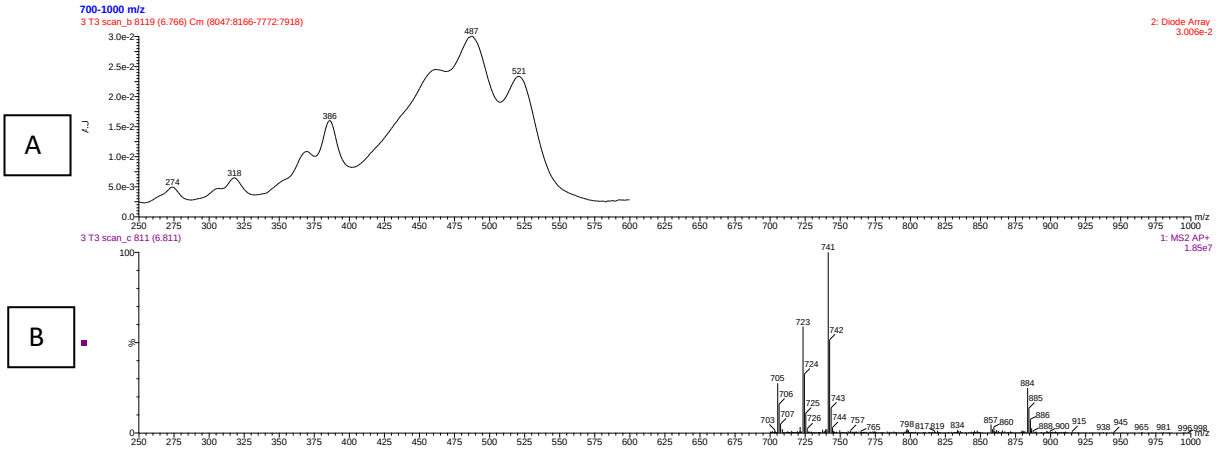
ESM 4. Peak 5.64 (A) UV/vis and (B) MS 700-1000 *m/z* spectra. The protonated molecule at 903 correspondent to bacterioruberin monoglucoside.



ESM 5. Peak 6.18 (A) UV/vis and (B) MS 700-1000m /z spectra. The protonated molecule at 903 correspondent to bacterioruberin monoglucoside, and the *cis peak* at 369 and 386 nm in the UV spectrum.

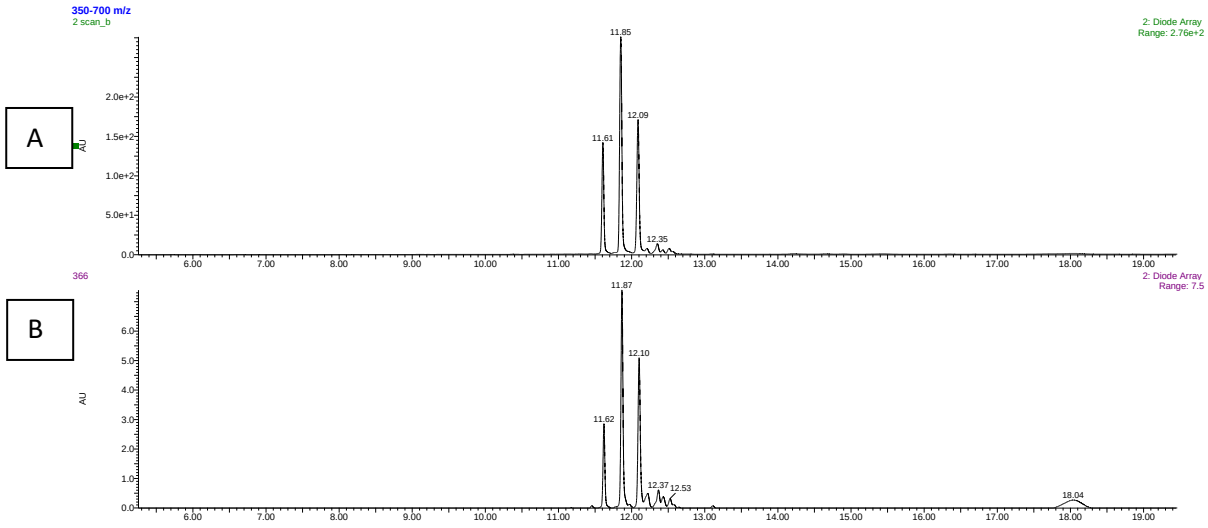


ESM 6. Peak 6.34 (A) UV/vis and (B) MS 350-700 *m/z*, (C) MS 700-1000 *m/z* spectra. The protonated molecule at 741 correspondent to bacterioruberin.

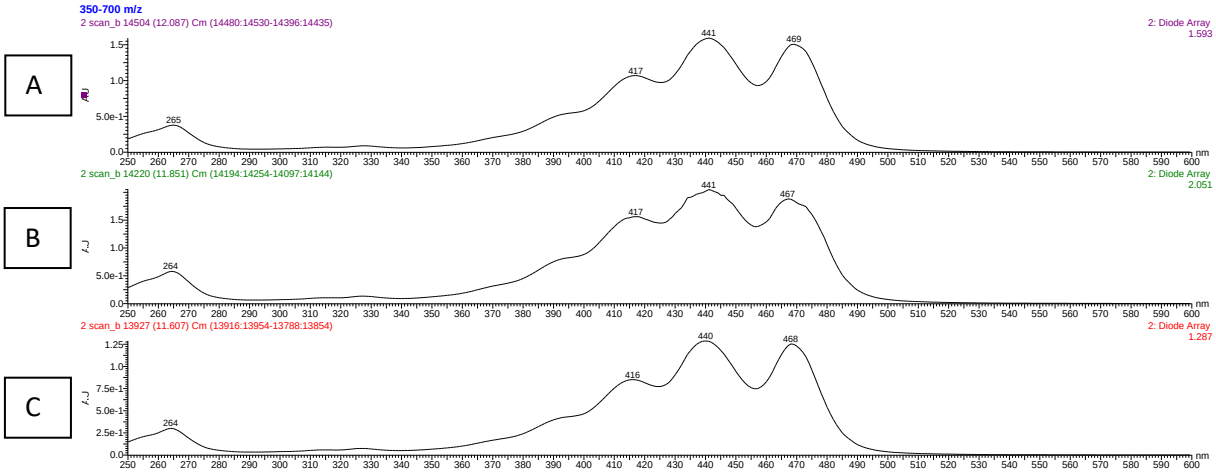


ESM 7. Peak 6.77 (A) UV/vis and (B) 700-1000 *m/z* spectra. The protonated molecula at 741 correspondent to bacterioruberin, and the *cis peak* at 386 nm in the UV spectrum.

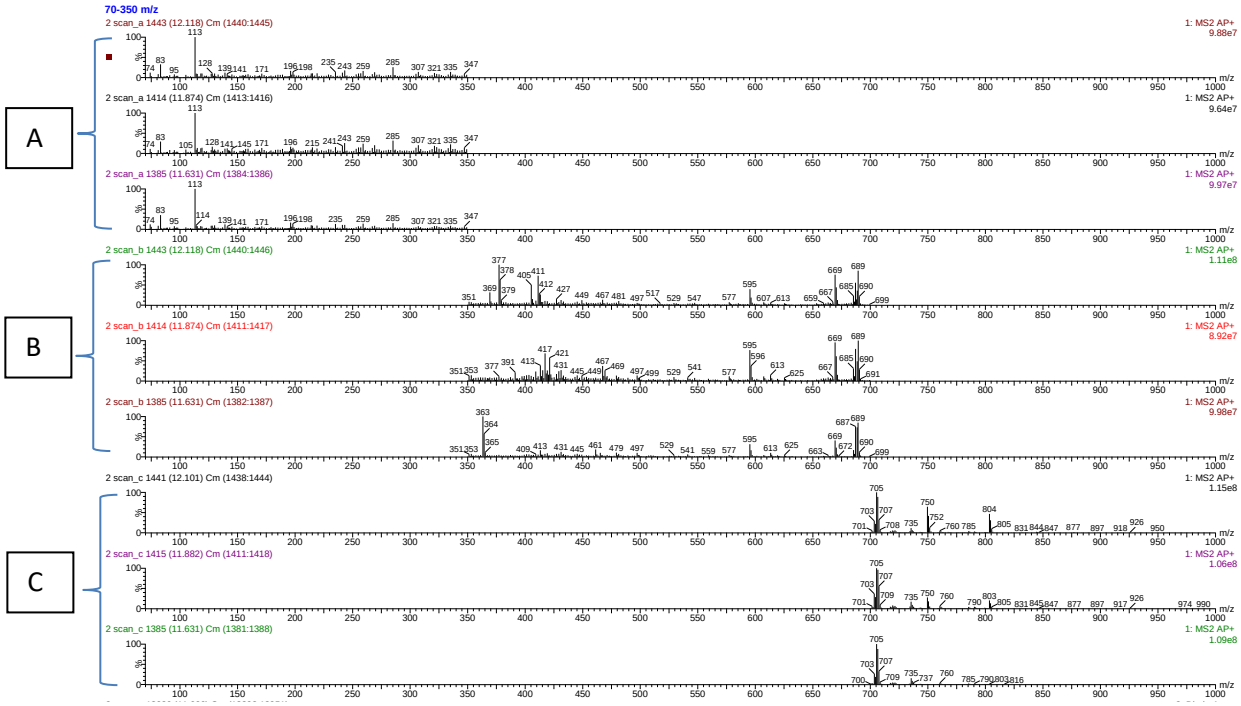
2- *Arthrobacter psychrochitiniphilus* 366



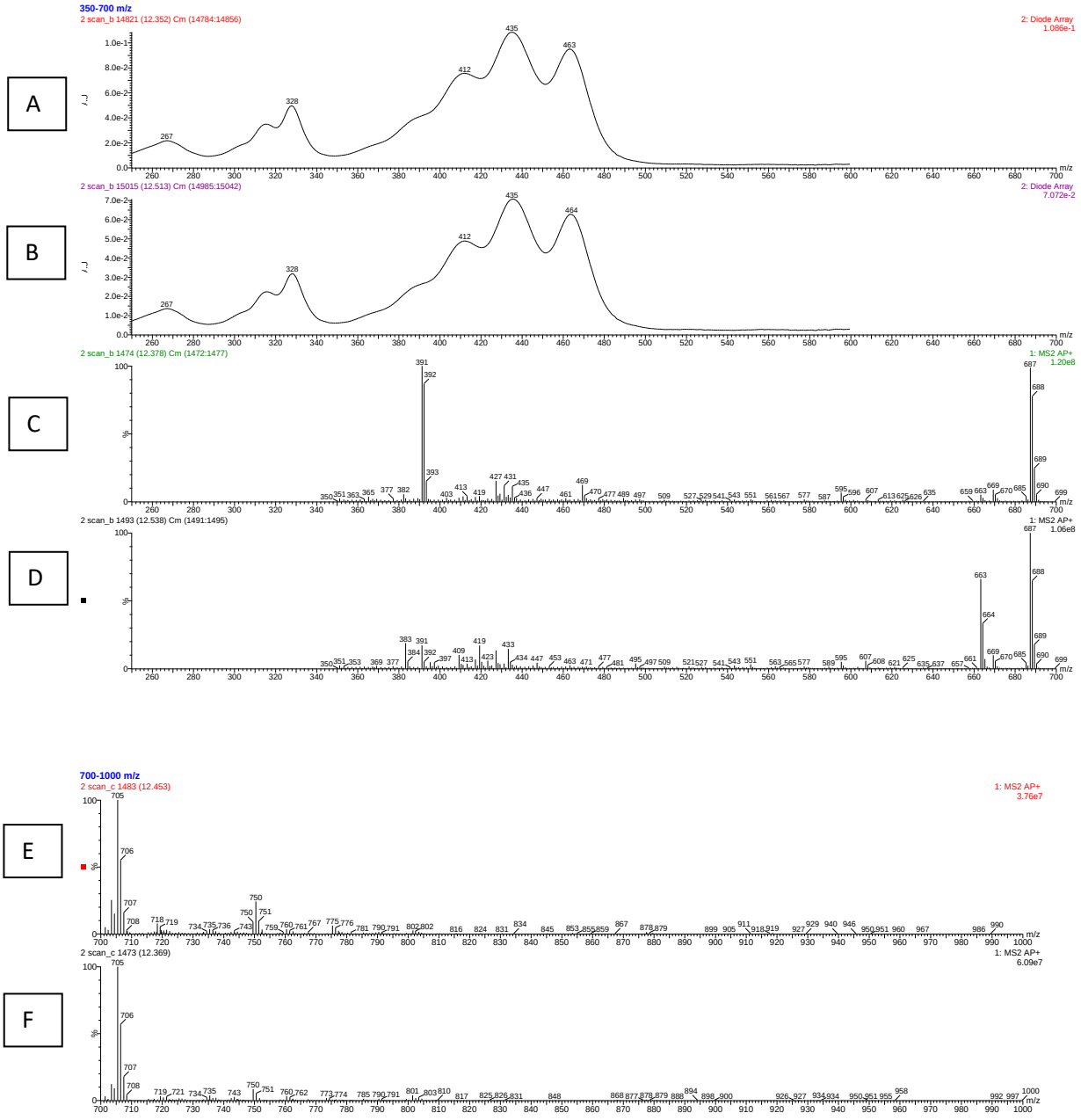
ESM 8. UPLC/PDA Chromatogram from (A) saponified and (B) non-saponified samples



ESM 9: UV/vis spectra from the peaks on retention time (A)12.08, (B)11.8 and (C) 11.6

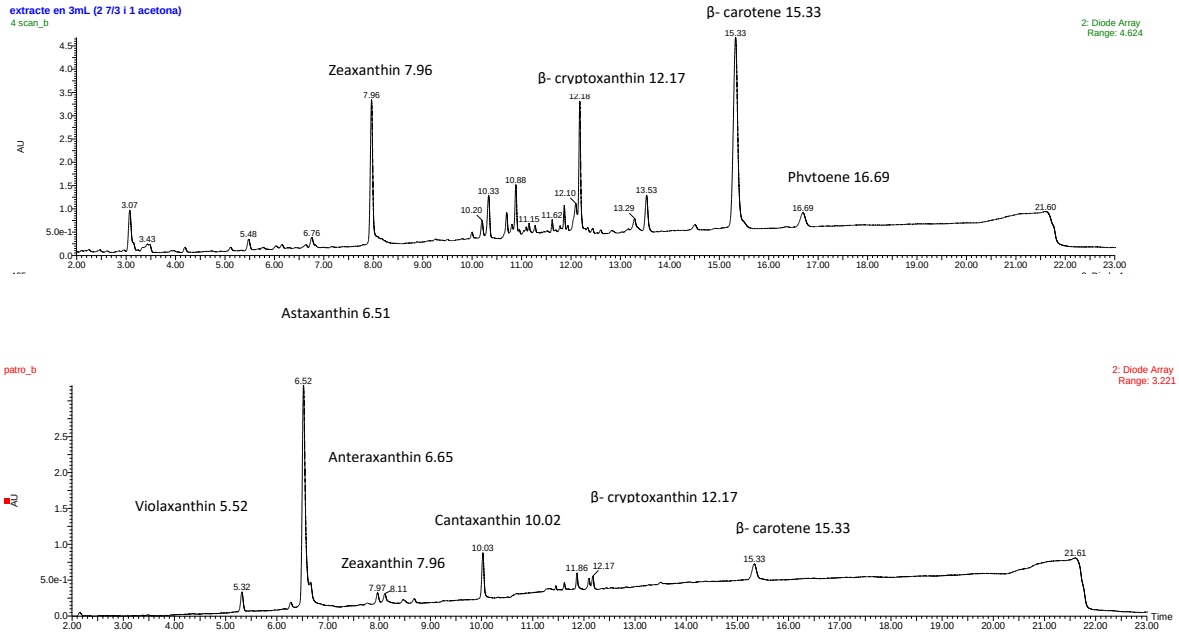


ESM 10: MS spectra from the peaks (A) 12.08, 11.8, 11.6 respectively at *m/z* 70-350 (B) 12.08, 11.8, 11.6 respectively at *m/z* 350-700 and (C) 12.08, 11.8, 11.6 respectively at *m/z* 700-1000.

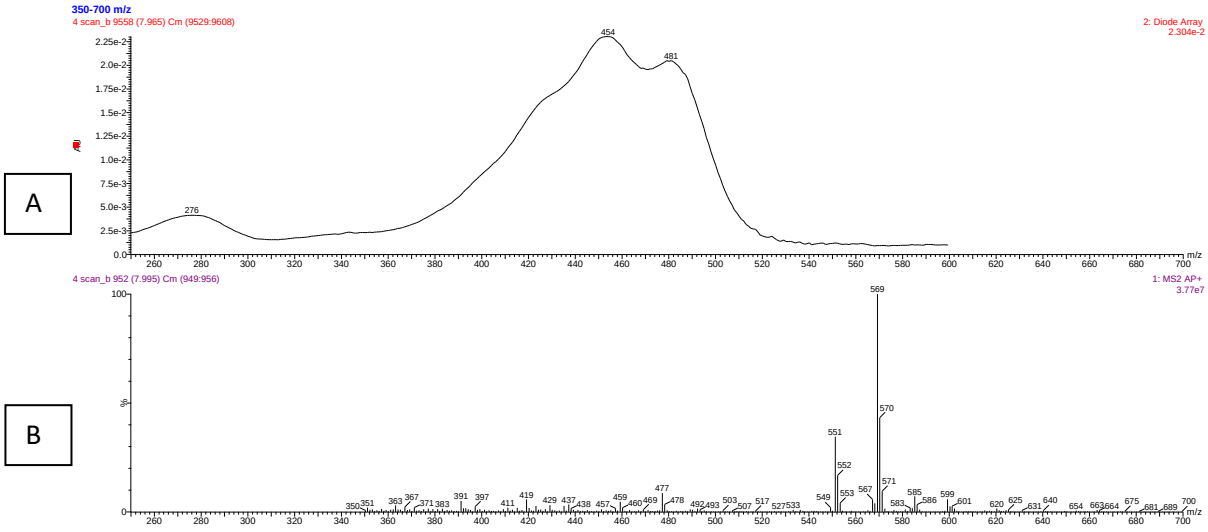


ESM 11: UV/vis spectra from the peaks on retention time (A) 12.35 and (B)12.51. MS spectra range 350-700 *m/z* (C) 12.35 and (D)12.51. MS spectra range 700-1000 *m/z* (E) 12.35 and (F)12.51. The UV-vis spectra present the *cis peak* at 328 for both samples.

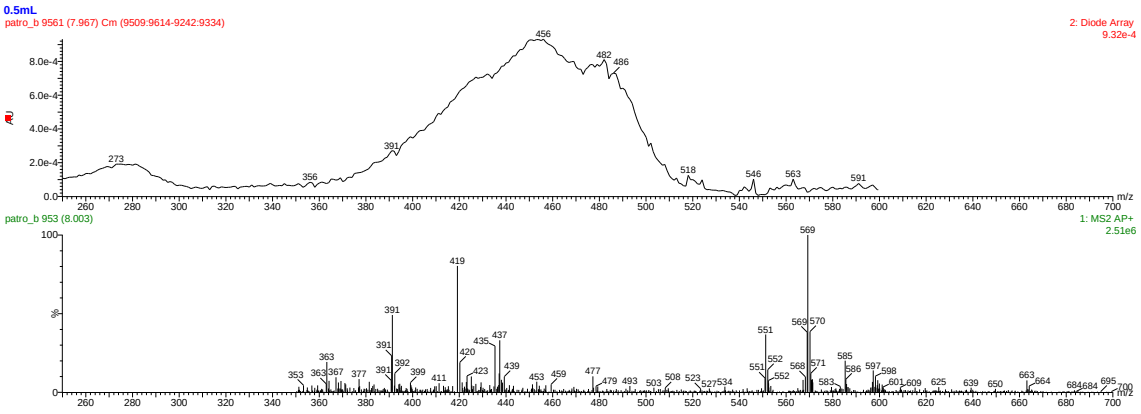
3- *Zobellia laminarie* 465



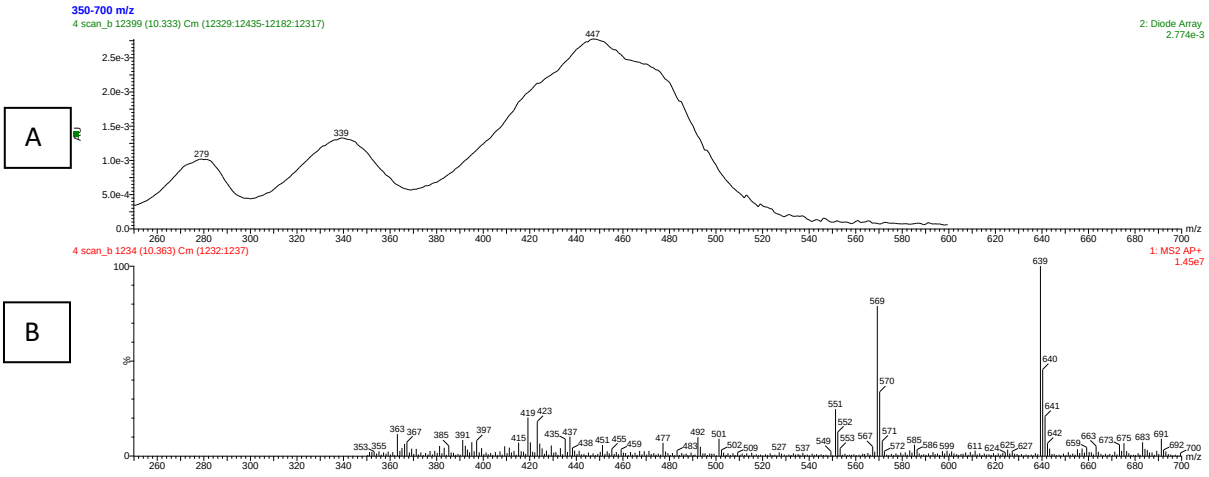
ESM 12. UPLC/PDA Chromatograns from (A) saponified 465 sample and (B) standards.



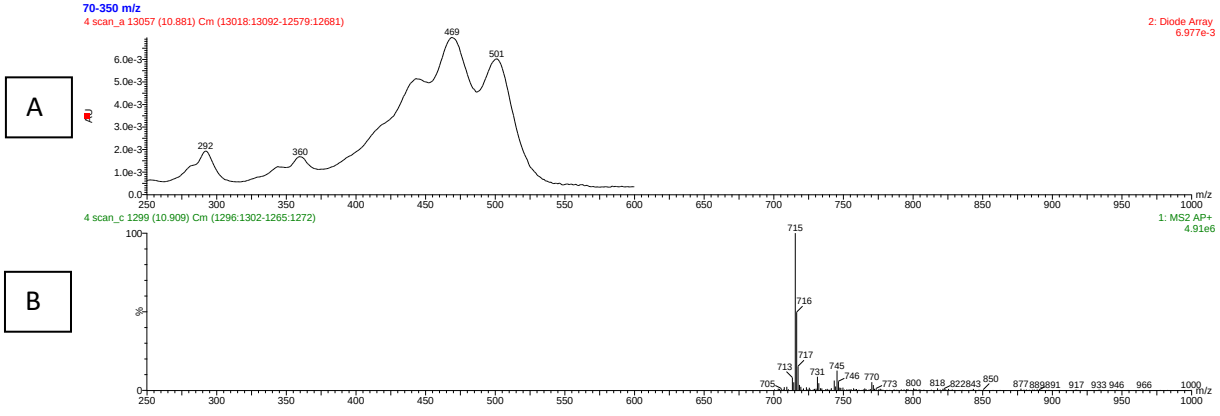
ESM 13. Peak on the retention time 7.96 (A) UV/vis and (B) 300-700 *m/z* spectra. The protonated molecule at 569 and the fragmentation at 551 *m/z* correspondent to zeaxanthin



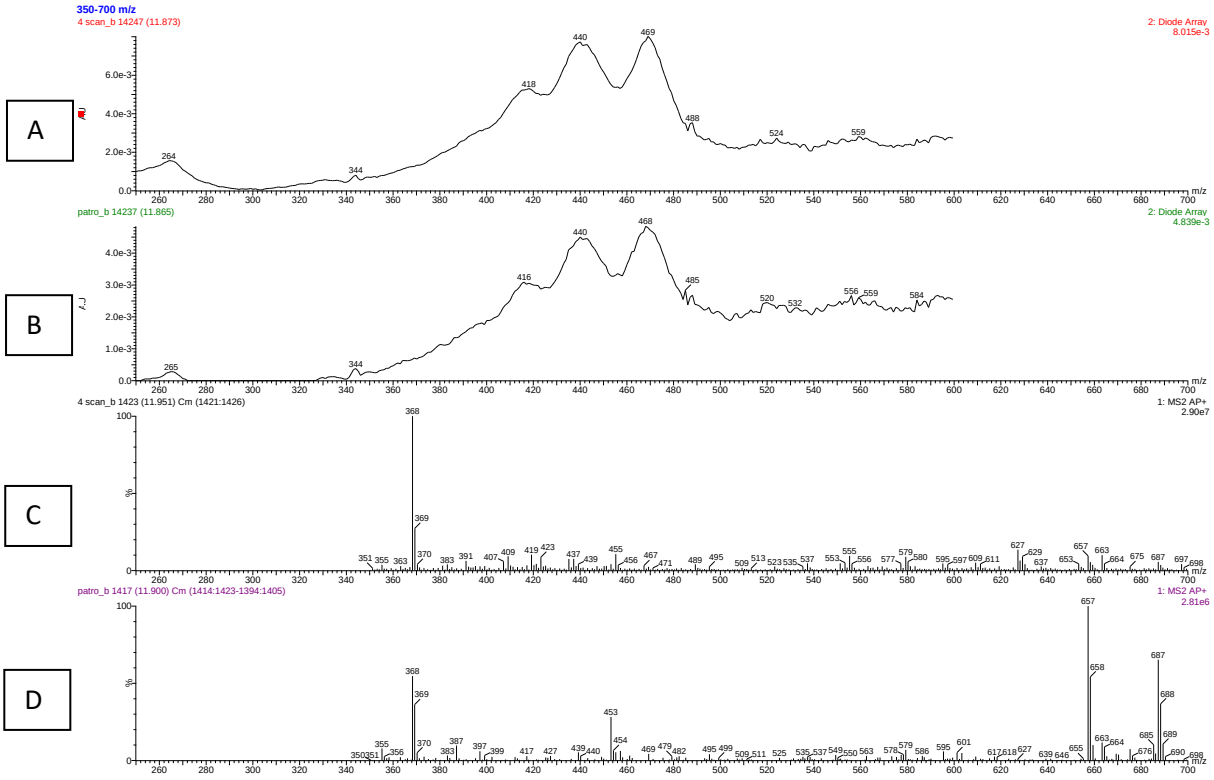
ESM 14. Peak 7.96 from **standard** (A) UV/vis and (B) 300-700 *m/z* spectra. The protonated molecule at 569 and the fragmentation at 551 *m/z* correspondent to zeaxanthin.



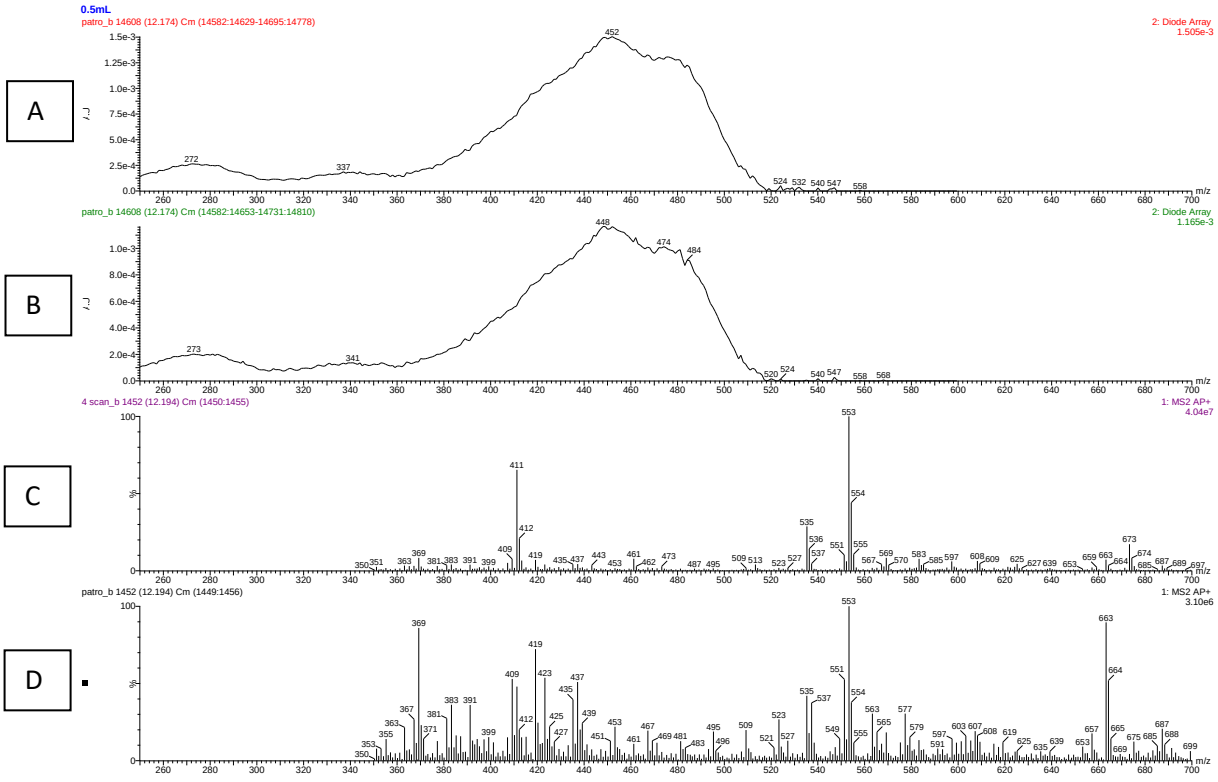
ESM 15. Peak 10.33 (A) UV/vis and (B) 300-700 *m/z* spectra. The protonated molecule at 639 was not identified. The UV spetrum is similar to semi Beta carotenone but the *m/z* is not correspondent.



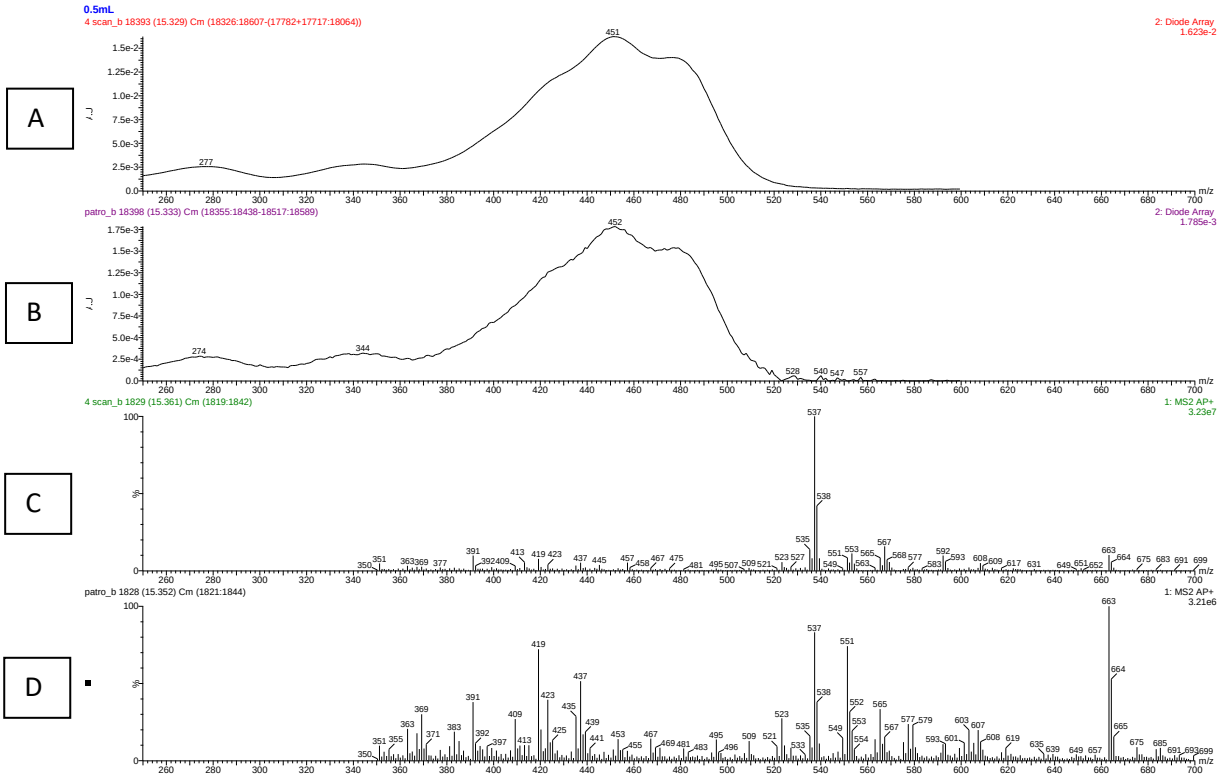
ESM 16. Peak 10.88 (A) UV/vis and (B) 300-700 *m/z* spectra. The protonated molecule at 715 was not identified. The UV spectrum is similar to CP-460.



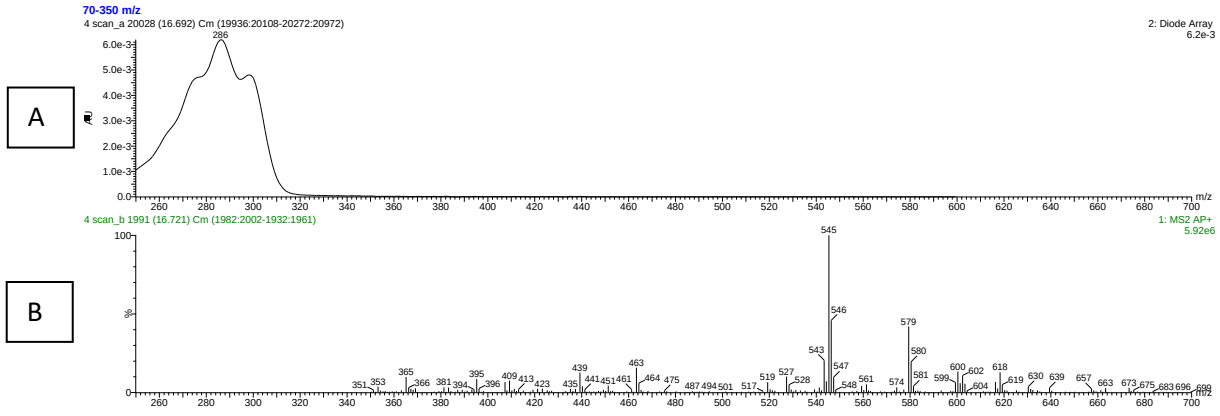
ESM 17. Peak 11.8 and 11.8 from standard. (A) and (B) UV/vis from 11.8 and 11.8 standard and (C) and (D) 300-700 *m/z* spectra. Unidentified carotene, probably some degradation from the other carotenes.



ESM 18. Peak 12.17: (A) and (B) UV/vis from peaks 12.17 and 12.17 standard respectively; (C) and (D) MS 300-700 *m/z* from peaks 12.17 and 12.17 standard respectively spectra. Identified as β-cryptoxanthin, protonated molecule *m/z* 553 and fragmentation at *m/z* 535.

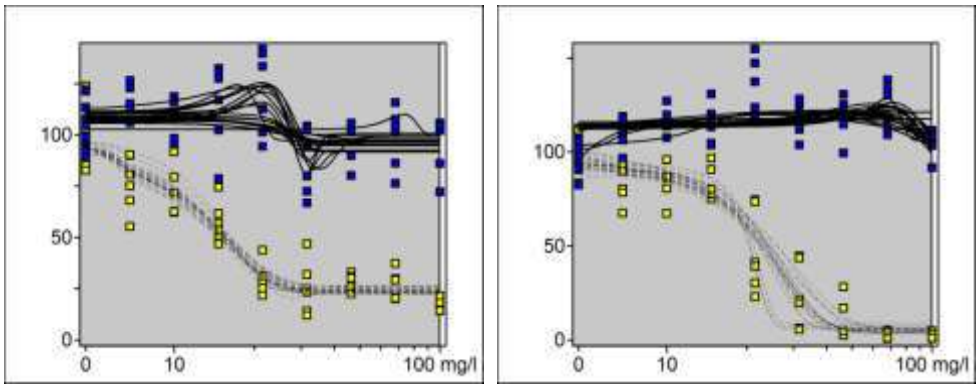


ESM 19. Peak 15.3: (A) and (B) UV/vis from peaks 15.3 and 15.3 standard respectively; (C) and (D) MS 300-700 *m/z* from peaks 15.3 and 15.3 standard respectively. Identified as β-carotene, protonated molecule *m/z* 538

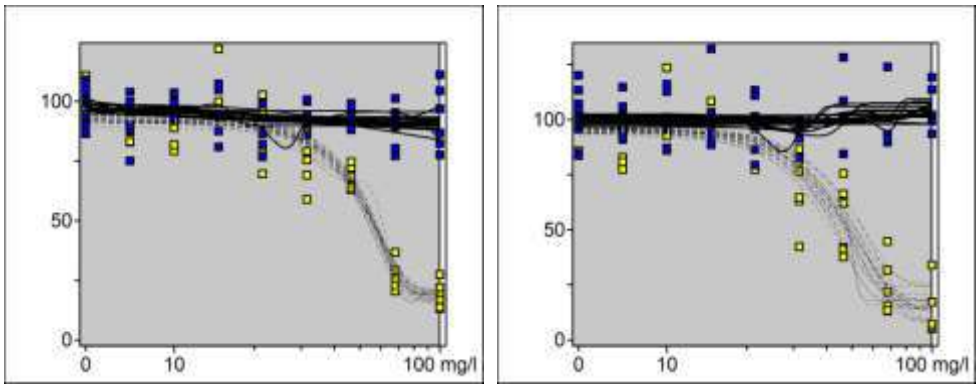


ESM 20. Peak 16.69: A) UV/vis and (B) 300-700 *m/z* spectra. The protonated molecule at 545 and the UV spectrum correspondent to phytoene.

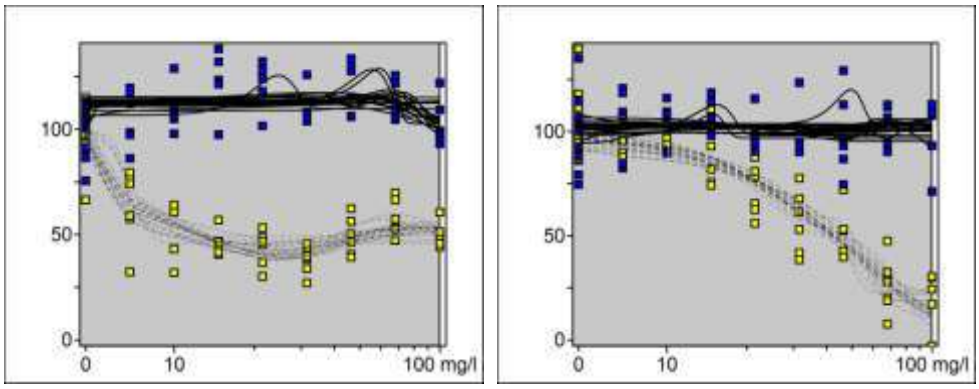
A **Pigment 465 fraction 35-40 min.** MPE: 0,559/ 0,676 Phototoxic IC50 +UV:15,4/ 23,18 µg/ mL.



B **Pigment 465 fraction 41-44min:**MPE: 0,145/ 0,285 Probably phototoxic/Phototoxic IC50 +UV:55,7 / 48,4 µg/ mL



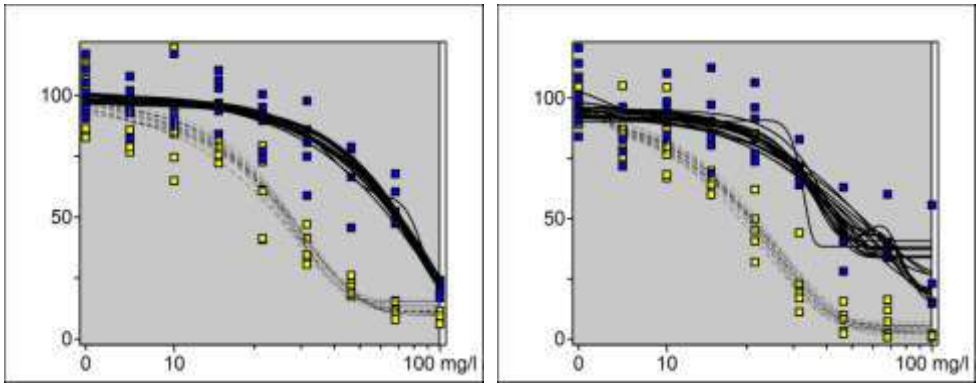
C **Pigment 465 fraction 56-59min:** MPE: 0,595/ 0,341 Phototoxic IC50 +UV:11,7 /43,47 µg/ mL.



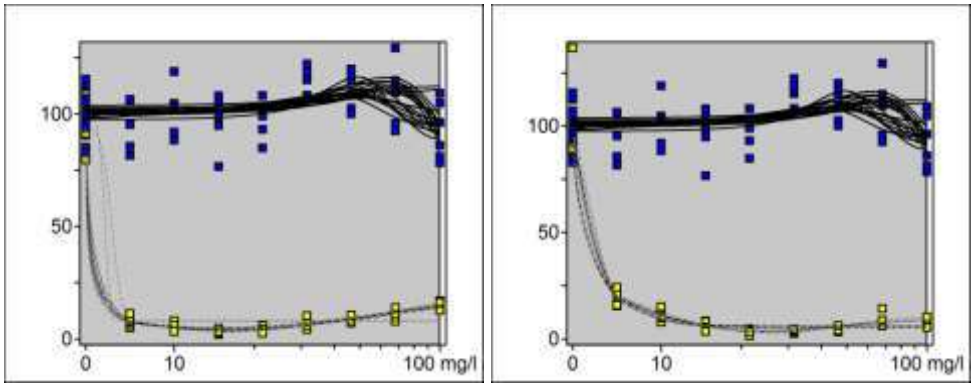
ESM 21**.** The dose–response curves (duplicate) of pigment (A) 465 fraction 35-40, zeaxanthin, (B) 465 fraction 41-44, β-cryptoxanthin and (C) 465 fraction 56-59, β-carotene; obtained by the 3T3 NRU phototoxicity test and plotted using the Phototox 2.0 software program. The blue (solid lines) and yellow squares (dotted line) refer, respectively, to non-irradiated substances (-

UV) and irradiated ones (+UV). Evaluated doses: 6.8, 10, 14.7, 21.4, 31.6, 46.4, 68.1 and 100 µg/mL.

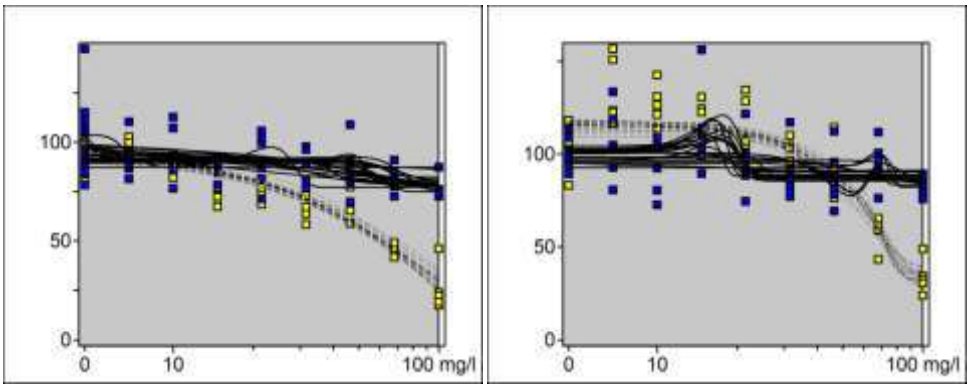
A **Pigment 50cyt fraction 18-21min:** MPE: - Cytotoxic IC50 +UV: 69,2 µg/ mLIC50-UV: 26,5 µg/ mL.



B **Pigment 50cyt fraction 23-24min:** MPE: 0.931/0,909 Phototoxic IC50 +UV: 0,7 e 2,6 µg/ mL.



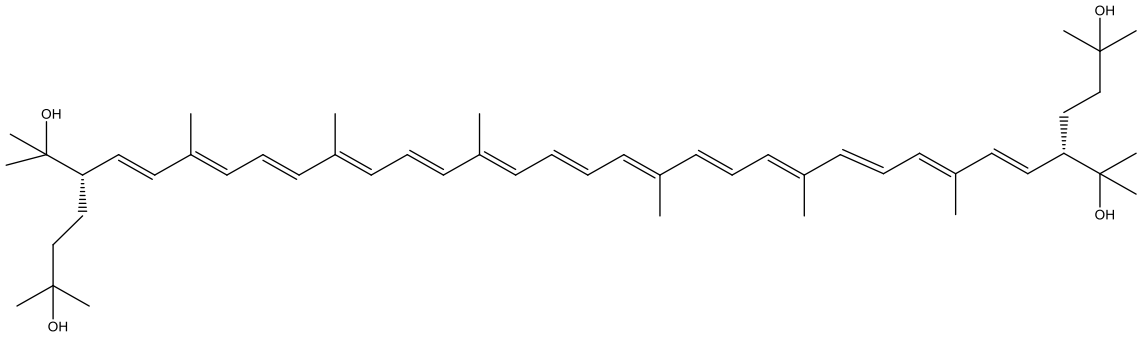
C **Pigment 50cyt fraction 28-29min:** MPE: 0,088/ -0,034 Non Phototoxic IC50 +UV: 61,59 e 74,30 µg/ mL.



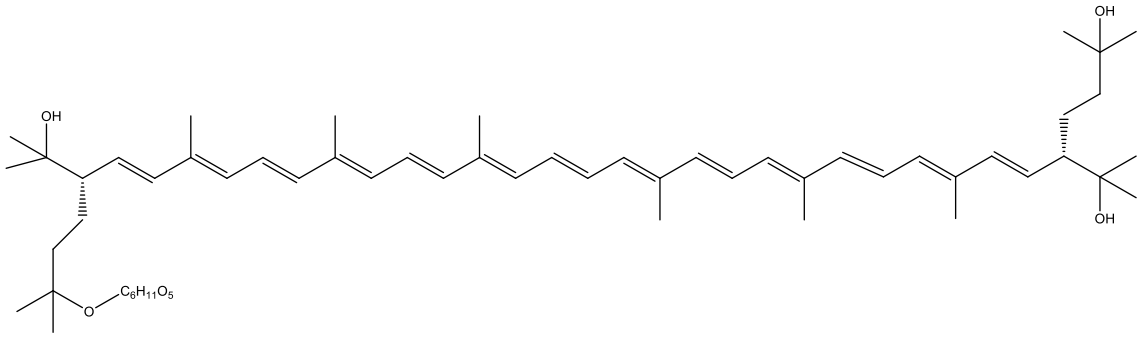
ESM 22**.** The dose–response curves (duplicate) of pigment (A) 50cyt fraction 18-21, bacterioruberin diglucoside, (B) 50cyt fraction 23-24, bacterioruberin monoglucoside and (C)

50cyt fraction 28-29, bacterioruberin; obtained by the 3T3 NRU phototoxicity test and plotted using the Phototox 2.0 software program. The blue (solid lines) and yellow squares (dotted line) refer, respectively, to non-irradiated substances (-UV) and irradiated ones (+UV). Evaluated doses: 6.8, 10, 14.7, 21.4, 31.6, 46.4, 68.1 and 100 µg/mL.

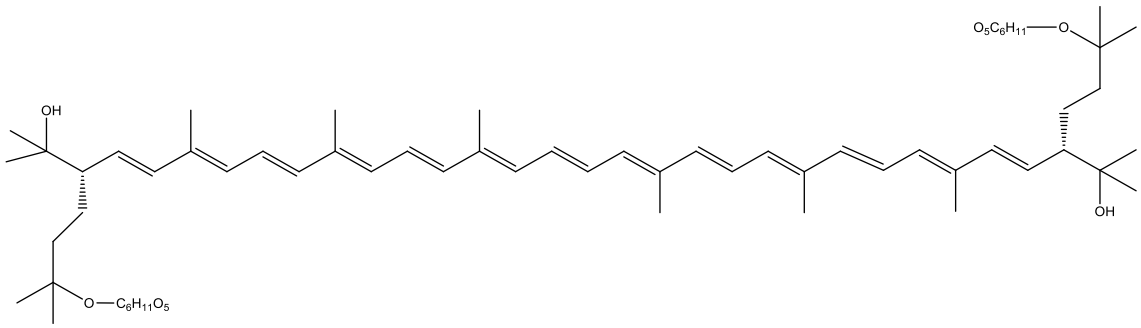
Bacterioruberin



Bacterioruberin monoglucoside

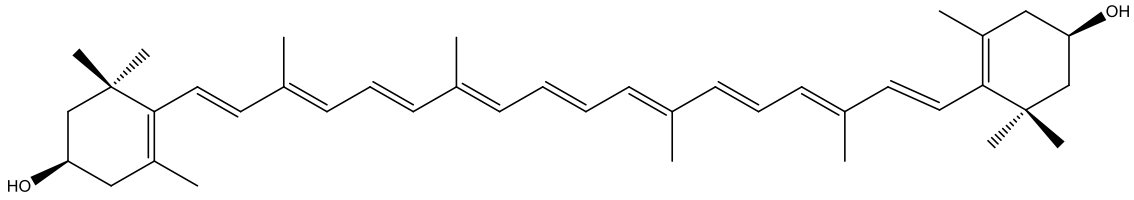


Bacterioruberin diglucoside

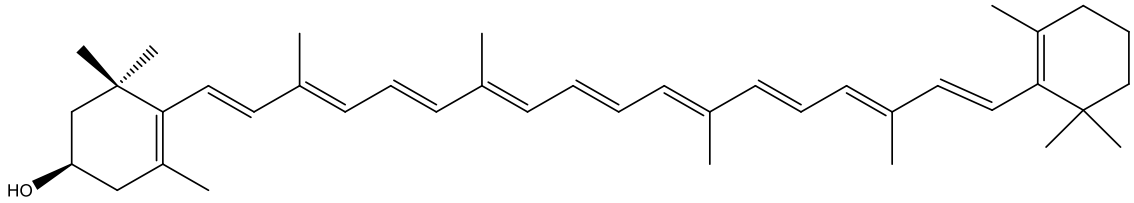


ESM 23: Chemical structure of C50 carotenoids bacterioruberin, bacterioruberin monoglucoside and bacterioruberin diglucoside isolated from *Arthrobacter agilis 50cyt*.

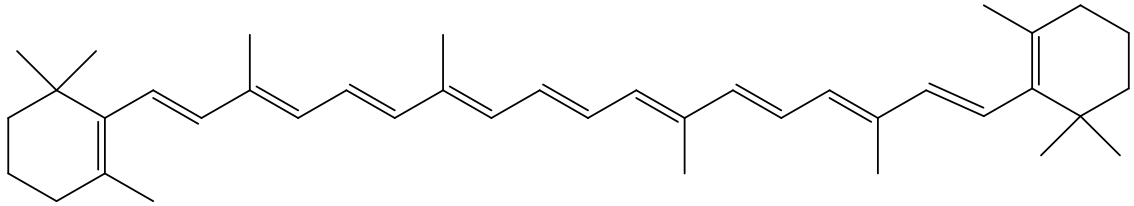
Zeaxanthin



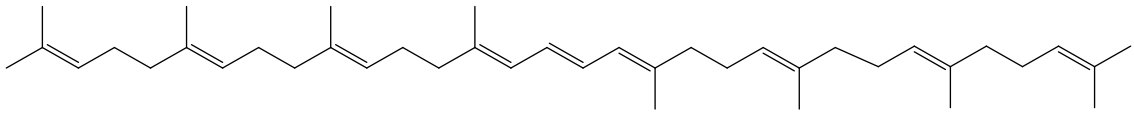
β –cryptoxanthin



β –carotene

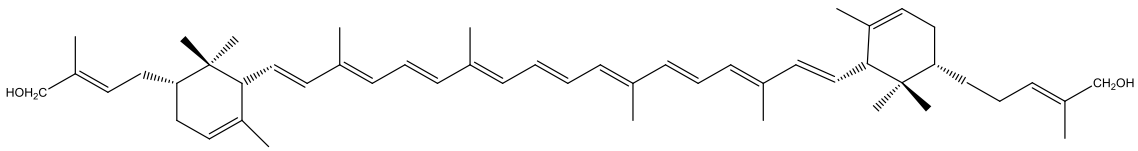


Phytoene



ESM 24: Chemical structure of C40 carotenoids – zeaxanthin, β –cryptoxanthin, β –carotene and phytoene isolated from *Zobellia laminarie* 465.

Decaprenoxanthin



ESM 25: Chemical structure of C50 carotenoid decaprenoxanthin isolated from *Arthrobacter psychrochitiniphilus* 366

CAPÍTULO III

Pigments in iridescent bacterium *Cellulophaga fucicola* isolated from Antarctica

Pigmentos da bactéria iridescente *Cellulophaga fucicola* isolada da Antártica

Antonie van Leeuwenhoek
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SHORT COMMUNICATION

Pigments in an iridescent bacterium, *Cellulophaga fucicola*, isolated from Antarctica

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Abstract An iridescent yellow pigmented bacterium isolated from the Antarctic continent, named *Cellulophaga fucicola* strain 416, was found to be able to tolerate UV-B radiation. Its crude pigment extract was tested for antioxidant capacity, UV light stability and phototoxicity profile against murine fibroblast lines. The pigments were further isolated and chemically identified by ultra-high-performance liquid chromatography with photodiode array and mass spectrometry detectors. The results showed that the pigment extract presented weak stability under exposure to UV light, a phototoxic profile in the 3t3 Neutral Red Uptake test and a very high antioxidant activity, suggesting that it could be used as food and feed colourants. Zeaxanthin and two isomers of zeaxanthin, β-cryptoxanthin and β-carotene, were identified using a C18 column. These five carotenoids were the major pigments isolated from *C. fucicola* 416. In conclusion, the identification of pigments produced by the bacterial strain under study may help us understand how bacteria thrive in high UV and cold environments, and opens avenues for further biotechnological application towards a more sustainable and environmentally friendly way of pigment exploitation.

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Introduction

Iridescence is an optical phenomenon of surfaces that appear to gradually change colour with the angle of observation and the angle of illumination. These functions are natural consequences of the colony structures formed, with periodic geometry, based on physical operation of light that interacts with different kinds of spatial inhomogeneity (Kinoshita et al. 2008). Gliding motility (without pili or flagella) is one of the factors responsible for the spatial arrangement of the colony resulting in iridescence phenomenon in bacteria (Kientz et al. 2016).

Examples of iridescence include soap bubbles, butterfly wings, hummingbirds, some minerals, and seashells. Iridescent bacterial strains are affiliated to different genera, most of them belonging to the phylum *Bacteroidetes*. Interestingly, the majority of isolated species have been associated with the marine flora (macroalgae) and fauna (mollusks and cnidarians) (Kientz et al. 2013). For several macroorganisms, recruitment of an epibiontal community with protection activities has been demonstrated (Patel et al. 2003; Goecke et al. 2010). Other authors proposed that the presence of iridescent bacteria in rocky shore ecosystems, that combine stressful conditions caused by solar exposure and desiccation, may suggest that iridescence and pigments could reflect specific wavelengths, such as UV and infrared (Doucet and Meadows 2009). This property could confer photoprotective and thermoregulatory functions for bacterial populations and/or their hosts in the environment (Kientz et al. 2013, 2016).

Bacterial pigments associated with iridescence have been poorly documented. The complete genomes of the iridescent bacterial species *Cellulophaga lytica* and *Cellulophaga algicola* have been sequenced demonstrating that they share the same genes coding for enzymes that are involved in the biosynthesis of carotenoids, e.g., phytoene desaturase and phytoene synthase (Abt et al. 2011; Pati et al. 2011). However, none of the pigments produced by iridescent bacteria has been identified to date.

Pigments from bacteria have a considerable potential to become novel bioproducts that could be used in several applications such as food colourants and in the pharmaceutical industry, textile industry and others (Ehling-schulz et al. 1997; Zollinger 2003; Malik et al. 2012; Venil et al. 2013). Pigments such as carotenoids have already been identified with photoprotective functions (Jagannadham et al. 2000). Therefore, pigments from iridescent bacteria represent a pool of unidentified colourants that could reveal interesting functions.

In this context, this work aimed to identify the pigments produced by the glitter-like iridescent bacterium *Cellulophaga fucicola* strain 416, previously isolated from a sea sponge in maritime Antarctica (Silva et al. 2018).

Materials and methods

The experimental strategy followed to achieve identification of the pigments from *C. fucicola* strain 416 is detailed in Fig. 1.

Bacterial strain

The bacterial strain 416 was previously isolated from a sea sponge collected during an expedition to maritime Antarctica in the austral summer (2013 and 2015) by the MycoAntar—Brazilian Antarctic Program team and identified as *C. fucicola* based on sequencing and phylogenetic analysis of the 16S rRNA gene (Silva et al. 2018).

Chemicals

The standards β-carotene, β-cryptoxanthin, zeaxanthin and astaxanthin were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Violaxanthin, canthaxanthin and antheraxanthin were purchased from Carotenature (Lupsingen, Switzerland). Methanol (MeOH), acetonitrile (ACN) and acetone (HPLC grade purity) were acquired from J.T. Baker (Deventer, The Netherlands). Water was purified in a Milli-Q reagent water system.

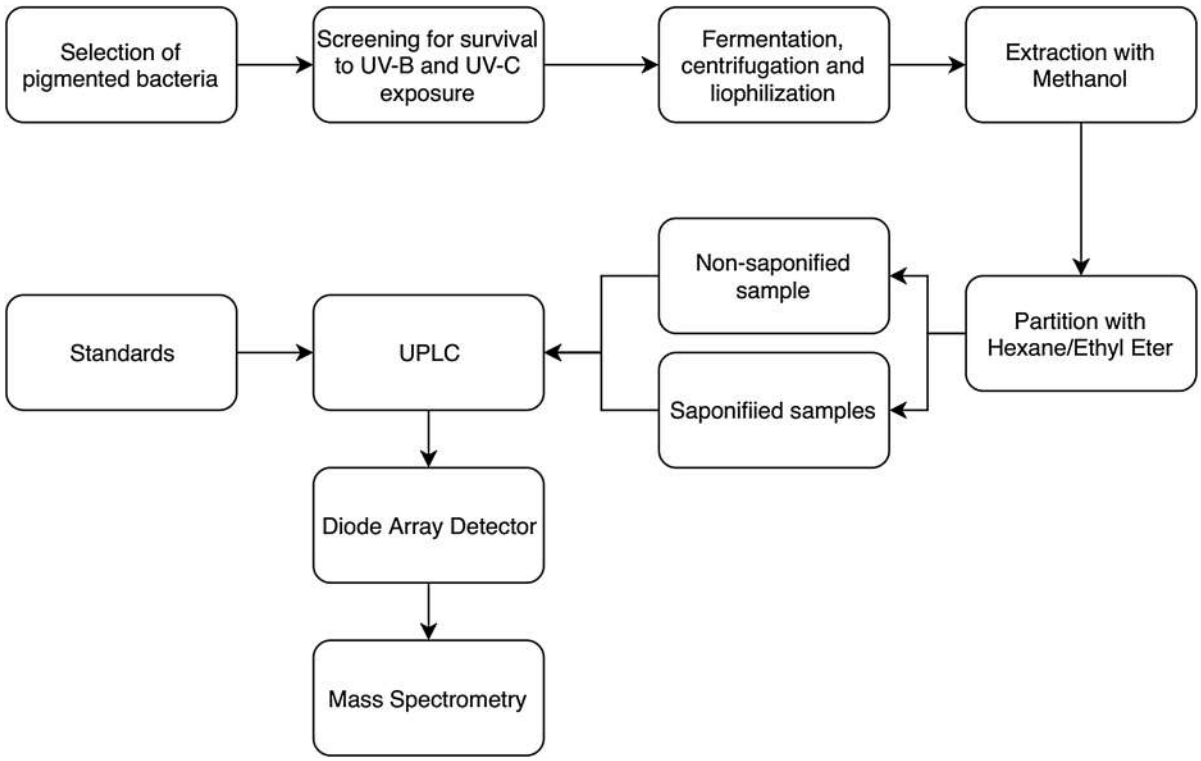

Fig. 1 Workflow of the basic strategy to reach pigment identification

Bacterial cultivation

One hundred µL-aliquots of bacterial cultures preserved in glycerol at − 80 °C were inoculated into 300 mL of Nutrient Broth culture medium to be used as inoculum for the shake flasks and the fermentation. Cultivation for production of biomass for further pigment characterisation was performed in 1 L-Erlenmeyer flasks filled with 600 mL Nutrient Broth medium diluted in artificial seawater (ASW). A total of 3000 mL of medium was used. Cultivation flasks were incubated for 7 days at 15 °C in a rotary shaker operated at 100 rpm. Aliquots of 50 mL were harvested by centrifugation at 8300 rpm ($8000\times g$) and the cell pellet was washed with 10 mL of distilled water and re-centrifuged. The cell pellet was freeze-dried and stored at − 20 °C prior to extraction of pigments.

Extraction of pigments and sample preparation

Lyophilised cells were extracted with 20 mL MeOH and vortexed for 5 min to ensure even dispersal of biomass. After centrifugation, the supernatant was collected, transferred to amber glass and concentrated under vacuum. The pigment was flushed with nitrogen and wrapped in aluminum foil to prevent light-mediated oxidation of the extracted carotenoids.

Before analysis, the crude extract of pigment was suspended in 4 mL of hexane and shaken for 10 min. Next, 2.3 mL of a solution of NaCl (10%, w/v) was added. The mixture was shaken for 15 min, cooled at 4 °C and the organic layer collected. The aqueous phase was re-extracted with 2 mL of hexane:diethyl ether (3:1, v/v) for 10 min and centrifuged at $2500\times g$ for 4 min. The organic solutions were combined in a 10-mL glass tube and dried under nitrogen stream at room temperature (Delpino-Rius et al. 2014).

Saponification

An aliquot of the dry residue was saponified in a shaking incubator for 60 min at 55 °C under N_2 using 1 mL of a 6% KOH solution in MeOH (w/v). After the addition of 1 mL of a solution of NaCl (10%, w/v), the

mixture was placed in the freezer for 15 min. Then, 2.5 mL of hexane:diethyl ether (3:1, v/v) was added, and the mixture was vortexed and centrifuged at 433 × *g* for 3 min (this step was repeated until the aqueous phase was colourless). The organic layers were combined and the solvent was removed under a nitrogen stream. The residue was stored at − 80 °C under argon atmosphere until UPLC analysis. The dry residue was dissolved in the injection solvent immediately before the analysis (Delpino-Rius et al. 2014).

Ultra-high-performance liquid chromatography with photodiode array and mass spectrometry (UPLC–PDA–APCI–MS/MS) analysis

The UPLC assay was carried out using an ACQUITY UPLC binary system. Mass detection was carried out using a Waters XEVO-TQD tandem quadrupole mass spectrometer (Manchester, UK). MassLynxTM software version 4.1 (Waters, Milford, MA) was used to control the instruments, and also for data acquisition and processing. UPLC chromatographic separations were performed on a reversed-phase column ACQUITY UPLCr BEH C18 130 Å, 1.7 µm, 2.1 × 150 mm (Waters). The mobile phase consisted of solvent A: acetonitrile (ACN): MeOH 7:3, v/v and solvent B: water 100%. The gradient program used is shown in Table 1. The column and sample temperatures were set at 32 and 25 °C, respectively. Injection volume was 5 µL. Optimised MS conditions are listed in Table 2. Each sample extract for LC analysis was dissolved in 1 mL of the injection solvent [ACN: MeOH 7:3, v/v]: acetone 2:1, v/v. Before use, all solutions were filtered through Millex 0.2 µm nylon membrane syringe filters (Millipore, Bedford, MA).

Table 1 Condition 1: gradient profile used in the separation of carotenoids by UPLC

Time (min)	Flow rate (mL/min)	A (%, v/v)	B (%, v/v)
Initial	0.5	75	25
1.03	0.5	75	25
9.88	0.5	95.1	4.9
11.38	0.7	100	0
20.18	0.7	100	0
21	0.5	75	25
23	0.5	75	25

Table 2 MS conditions

MS conditions	APCI
Polarity	Positive
Corona (kV)	4.0
Cone (V)	30
Extractor (V)	3
RF (V)	0.1
Source temperature (°C)	150
Probe temperature (°C)	450
Cone gas flow (L/h)	10
Desolvation gas flow (L/h)	150
Collision gas flow (mL/min)	0.15

Evaluation of photostability by UV spectrometry

The extracted pigment was submitted to duplicate photodegradation studies. The photostability was determined by the comparative analysis of the absorption spectrum of the pigments in solutions at 200 µg/mL in the Gehaka model UV-380G spectrometer (São Paulo, Brazil) in the range of 200–400 nm, which was subjected to an UVA irradiance close to 8.3 mW/cm^2 emitted by a Philips UVA Actinic BL/10 lamp (Eindhoven Netherlands) for 52 min, giving a total dose of 27.5 J/cm^2 (Whitehead and Hedges 2005; Gaspar and Maia Campos 2006) compared to those who were left out of the light.

The determination of photostability was achieved by the ratio of the integrating area under the curve of the irradiated spectrum in the range of UVB (280–320 nm), UVA (320–400 nm) and visible (400–700 nm) compared with the integrating area of the non-irradiated absorption spectrum in the UVB, UVA, and visible ranges.

Phototoxicity test in cell culture

The 3T3 Neutral Red Uptake (NRU) Phototoxicity Test was performed according to INVITTOX Protocol No. 78 (Spielmann et al. 1998; Ecvam Db-Alm 2008), using 3T3 Balb/c fibroblasts (L1, ECACC No. 86052701). For this purpose, after evaluation of the fibroblasts sensibility to the UVA radiation, two 96-well plates were used for the pigment test, one to determine the cytotoxicity (absence of radiation, − UVA plate) and the other for phototoxicity

(presence of radiation, + UVA plate). For that, firstly 100 µL of a cell suspension of 3T3 fibroblasts in Dulbecco's Modification of Eagle's Medium containing New Born Calf Serum and antibiotics (1×10^5 - cells/mL, 1×10^4 cells/well) was dispensed in two 96-well plates. After a 24 h period of incubation (7.5% CO_2, 37 °C), plates were washed with 150 µL of Earle's Balanced Salt Solution (EBSS) and 8 different concentrations (6.81 µg/mL, 10 µg/mL, 14.7 µg/mL, 21.5 µg/mL, 31.6 µg/mL, 46.4 µg/mL, 68.1 µg/mL and 100 µg/mL) of the test chemicals or combinations were applied in sextuplicate in the 96-well plates. After 1 h incubation, the + UVA plate was irradiated for approximately 20 min with 7 mW/cm^2 achieving 9 J/cm^2 of UVA radiation from a UV-sun simulator, type SOL-500 (Dr. Hönle, Germany). In the meantime, the UVA plate was kept in a dark box. Culture medium replaced the test solutions and the plates were incubated overnight. Neutral Red medium was added in each well and after an incubation period, cells were washed with Dulbecco's phosphate-buffered saline and a desorbent (ethanol/acetic acid) solution was added. Neutral red extracted from viable cells formed a homogeneous solution and the + UV and − UV plates were analysed in a microplate reader at 540 nm.

For concentration–response analysis, Phototox Version 2.0 software (obtained from ZEBET, Germany) was employed. A test substance is predicted as having a potential phototoxic hazard if the photoirritation factor, calculated as the ratio of toxicity for each substance with and without UV light, is higher than 5 (Spielmann et al. 1998). Using the Phototox software, a second predictor of phototoxicity, the mean photoeffect (MPE) was also calculated. The MPE is a statistical comparison of the dose–response curves obtained with and without UV (Holzhutter 1997). According to the Organisation for Economic Cooperation and Development (OECD) Test Guideline 432, a test substance with MPE values below 0.1 is predicted to be "non-phototoxic", values between 0.1 and 0.15 are predicted to be "probably phototoxic" and values greater than 0.15 predict "phototoxic" (OECD 2004; Kejlová et al. 2007). Results are the mean of at least two independent experiments (± SEM). Norfloxacin and L-histidine were used as positive and negative controls, respectively.

Antioxidant activity

DPPH˙ scavenging assay

Free radical-scavenging activity was measured using a method adapted by Brand-Williams et al. (1995). Experiment was performed on freshly prepared ethanolic solutions of 2,2-diphenyl-1-picrylhydrazyl (DPPH; 0.004% w/v). In brief, 50 µL of extract in different concentrations was mixed with 250 µL of DPPH solution in a transparent microplate with 96 wells (Costar, Cambridge, MA). After 30 min of reaction, the absorbance of the remaining DPPH was measured at 517 nm on a microplate reader NOVOstar (BMG Labtech®, Offenburg, Germany). Antioxidant activity was expressed as a percentage of the absorbance of the control DPPH solution, obtained from the following equation: % Activity = $[(A_{DPPH\cdot} - A_{sample})/A_{DPPH})] \times 100$, where A_{DPPH} is the absorbance value of the control, and A_{sample} is the absorbance value of the test solution. Trolox was used as a standard to generate a calibration curve (15–250 µM), and the results were expressed as µmol Trolox equivalent (TE)/mg of pigment. These results were obtained in triplicate and expressed as mean ± SD.

ABTS˙$^+$ scavenging capacity assay

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS˙$^+$) scavenging capacity assay was determined as described by Le et al. (2007). The method is based on the decolourisation of the ABTS radical cation to determine the antioxidant potential of samples. The solution of ABTS radical cation was prepared in advance by reacting aqueous ABTS solution (7 mM) with potassium persulfate (2.45 mM). In the analysis, diluted ABTS˙$^+$ solution with an absorbance of 0.70 ± 0.02 at 734 nm was employed. The assay was performed using quartz cuvettes with a black mask and the reaction system was composed of 200 µL of sample and 1000 µL of ABTS˙$^+$solution, followed by 6 min incubation at room temperature. The absorbance values were measured on a spectrophotometer (DU 640, Beckman, Coulter Inc., CA, USA) at 734 nm in triplicate. Free radical scavenging activity was expressed as a percentage of the absorbance of the control ABTS˙$^+$, obtained with the following equation: % Activity = $[(A_{ABTS+}^{\cdot} - A_{sample})/A_{ABTS+}^{\cdot}] \times 100$,

where A_{ABTS+} is the absorbance value of the ABTS$^{\cdot}$ control, and A_{sample} is the absorbance value of the extract solution. A calibration curve was plotted of absorbance reduction and concentration of the Trolox (10–250 μM) and the results were expressed as μmol TE/g of pigment. These results were obtained in triplicate and expressed as mean ± SD.

Oxygen radical absorbance capacity ($ORAC_{FL}$) assay

The $ORAC_{FL}$ assay was described by Prior et al. (2003) and modified by Dávalos et al. (2004). The reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 200 μL. Antioxidant sample (20 μL) and fluorescein (120 μL; 70 μM in final concentration) solutions were mixed in one of the 96 wells of a black microplate (Costar, Cambridge, MA). Then, 60 μL of an 2,2′-Azobis(2-amidinopropane) dihydrochloride solution (final concentration 12 mM) were added and fluorescence was checked every cycle of 60 s for 80 cycles. The automated ORAC assay was performed on a NovoStar Microplate reader (BMG Labtech, Offenburg, Germany) with fluorescence filters (excitation, λ 485 nm; emission λ 520 nm). The experiment was conducted at 37 °C and pH 7.4 with a blank sample in parallel. The result was calculated using the differences of areas under FL decay curves between the blank (net AUC) and sample and was expressed as equivalent for μmol of Trolox (μmol/TE/g) per μmol of pigment, as described in the following equation: $AUC1 + \Sigma f_i/f_0$, where f_0 is the initial fluorescence (t = 0) and f_i is the fluorescence obtained at t = i (min). Net $AUC = AUC_{sample} - AUC_{blank.}$

Net AUC was plotted against sample concentration and results were compared to the standard curve (Net AUC versus Trolox concentration). The equivalence of Trolox was given by the angular coefficient of Trolox curve concentration (μM) versus sample concentration (μM). All assays were performed in three independent replicates.

Results

Identification of pigments

Supplementary Fig. 1 shows the bright aspect of *C. fucicola* 416, which was observed macroscopically under oblique direct illumination. The dominant glitter-like colours were observed to be yellow at the colony center and green at the colony peripheral edges. The PDA/UPLC chromatogram of pigment from *C. fucicola* 416 is presented in Fig. 2a and the chromatogram of the standards is shown in Fig. 2b.

The characteristics of the pigments separated in the UPLC/MS system are shown in Table 3. The chemical structure of the identified carotenoids is presented in Supplementary Fig. S2. The identification was achieved according to the retention time (RT) in the C18 column, in relation to the UV and MS spectra (Figs. S2, S3, S4 and S5).

(a) *Zeaxanthin* (peak 7.93 min) (Fig. S3) was identified as *all-trans*-zeaxanthin considering the UV–visible spectrum, λmax at 453 and 479 nm, defined fine spectral structure (III/II) 86%, mass spectrum characteristics and confirmed by coelution with the *all-trans*-zeaxanthin standard. As expected, the mass spectrum showed the protonated molecule at *m/z* 569 and fragments at *m/z* 551 $[M + H\text{-}18]^+$.

(b) *Zeaxanthin isomers* (peaks 10.19 and 10.31) (Fig. S4): The identification of the two *zeaxanthin* minor isomers was indicated by their mass spectra with the protonated molecule at *m/z* 569 and fragments at *m/z* 551 $[M + H\text{-}18]^+$. The UV spectrum showed characteristic UV–visible spectrum, with a hypsochromic shift of 5 nm for the 10.19 isomer and hypsochromic shift of 6 nm for the *cis*-isomer 10.31 with the *cis* peak at 339 nm.

(c) *β-cryptoxanthin* (peak 12.17) (Fig. S5): Identified as *all-trans*-β-*cryptoxanthin*, with the UV–visible spectrum similar to those from zeaxanthin with maximum absorbance at 452 nm. The protonated molecule was detected at *m/z* 553, along with less intense fragments at *m/z* 535 $[M + H\text{-}18]^+$ and 461 $[M + H\text{-}92]^+$. The identification was confirmed through co-elution with the *all-trans*-β-cryptoxanthin standard.

(d) *β-carotene* (peak 15.33) (Fig. S5): Identified as *all-trans*-β-carotene, with the UV–visible spectrum similar to those from zeaxanthin with maximum absorbance at 451 nm. The protonated molecule was detected at *m/z* 538, along with less intense fragments at *m/z* 444 $[M + H\text{-}92]^+$. The identification was confirmed through co-elution with the *all-trans* standard.

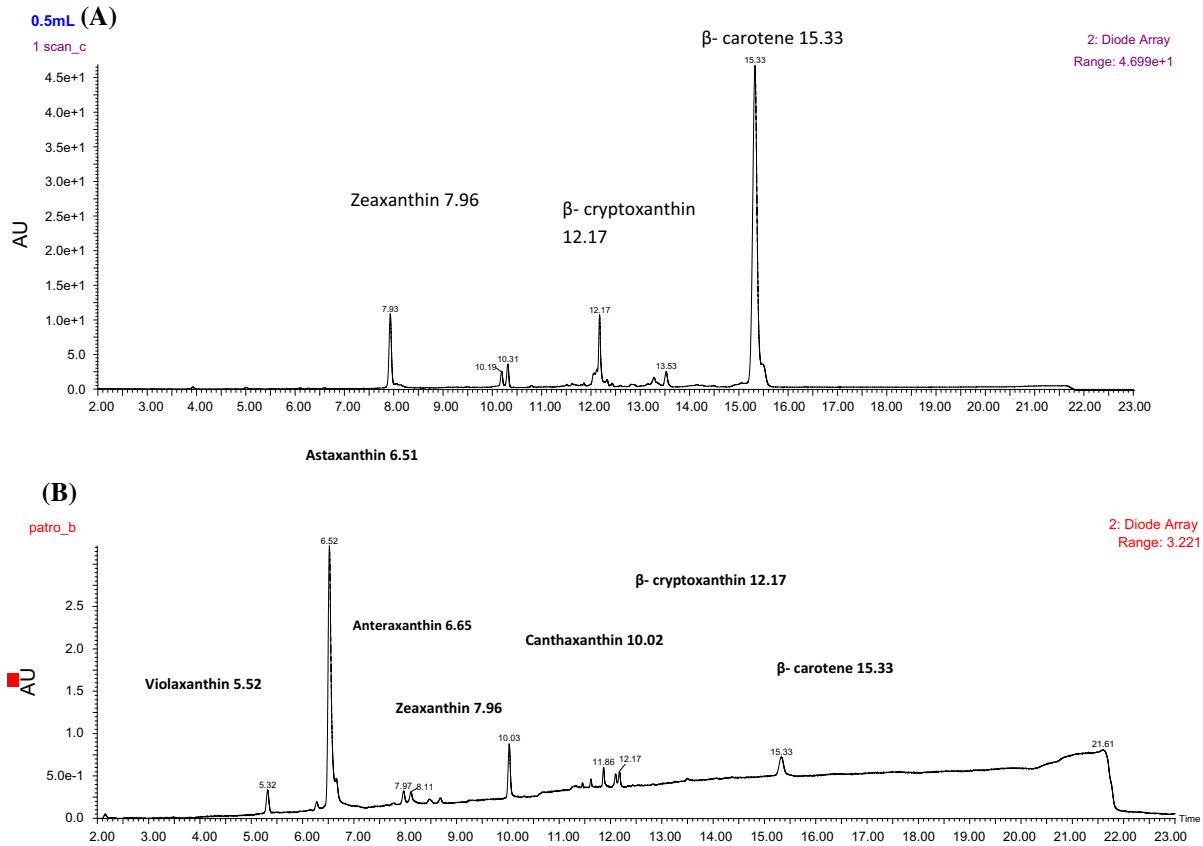

Fig. 2 UPLC/PDA (250–600 nm) chromatograms performed in the same conditions **a** saponified 416 sample, and **b** carotenoid standard mixture. Standard Rt were as follows: Violaxanthin 5.31, Astaxanthin 6.51, Anteraxanthin 6.65, Zeaxanthin 7.96, Lutein 8.11, Canthaxanthin 10.02, β-Cryptoxanthin 12.17 and β-Carotene 15.33

Table 3 Summary of the chromatographic, UV–vis and MS parameters of carotenoids found in *C. fucicola* 416

Retention time (min)[a]	Carotenoid	λ_{max} (nm)[b]	$[M + H]^+$(m/z)	Fragment ions (*m/z*)	%III/II[c]
7.93	Zeaxanthin	453, 479	569	551 [M + H-18]	86
10.19	Zeaxanthin isomer	448, 475	569	551 [M + H-18]	88
10.31	*cis*-Zeaxanthin	Cis (339), 447, 475	569	551 [M + H-18]	83
12.17	β-Cryptoxanthin	452	553	535 [M + H-18], 461 [M + H-92]	87
15.33	β-Carotene	451	538		86

[a]Retention time on the C18 column

[b]Linear gradient of ACN:MeOH/water

[c]Defined spectral fine structure

Photostability, phototoxicity tests and antioxidant capacity

In a screening for cell survival against UV-B and UV-C radiation, the bacterium *C. fucicola* 416 presented resistance to UV-B when compared to *Escherichia coli* and other pigmented bacteria, but not resistance against UV-C (data not shown). Subsequently, the pigment was extracted and analysed for photostability (Fig. 3; Table S1) and phototoxicity (Fig. S7). The

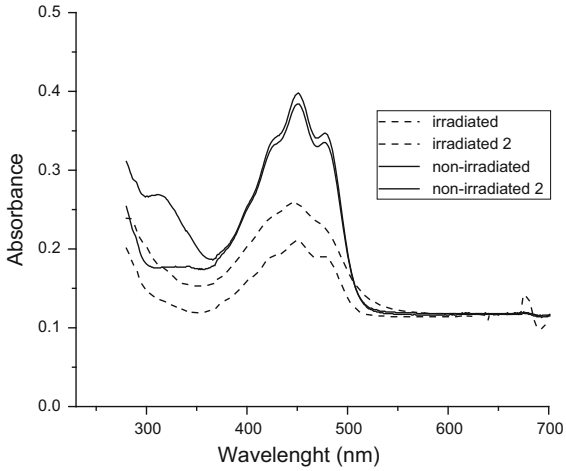

Fig. 3 Photostability of crude extract pigment from *C. fucicola* 416 after 52 min exposure at UV-A radiation. The solid line represents the non-irradiated pigment and the dotted line represents the pigment after irradiation

Table 4 Results of 3T3 NRU phototoxicity test

Sample	Phototoxicity	MPE[b]
Norfloxacin (C +)[a]	Phototoxic	0.615/0.909
C. fucicola 416	Phototoxic	0.461/0.143

[a]Norfloxacin used as positive control

[b]Mean photoeffect—MPE < 0.1 non-phototoxic; MPE > 0.1 and < 0.15: probably phototoxic; MPE > 0.15: phototoxic

spectrum of the crude pigment extract showed absorbance in a range between long UVA and Visible light. It presented some remaining absorbance after UVA irradiation in comparison with the spectrum of the extract in absence of irradiation. Figure 3 summarises the results obtained. After 52 min of irradiation the remaining pigment (area under the curve maintained) was 76.5% in UVB, 73% in UVA and 79% in visible light. The pigment extract was not considered photostable as the cutoff is a 20% decrease (Gaspar and Maia Campos 2006).

According to the results obtained in the 3T3 NRU test in fibroblasts from mice (Table 4 and Fig. S7), the pigment was considered phototoxic since it presented a mean MPE of 0.461. This means that the pigment did not pass in this preliminary test for usage as a photoprotective compound for skin.

The crude extract of pigment from *C. fucicola* 416 was tested for different antioxidant assays, the results obtained are expressed in µmol Trolox equivalent (TE)/mg of pigment: 3.73 ± 0.11 in DPPH; 3.56 ± 0.67 in ABTS-H; 3.82 ± 0.42 in ABTS-L; 1.36 ± 0.03 in ORAC-H; 5.12 ± 0.41 in ORAC-L; and 6.48 in ORAC-CAT (Table 5).

Discussion

The analysis of pigments from the iridescent bacterium *C. fucicola* strain 416 fulfilled the three minimum recommended procedures for identification and structure elucidation of carotenoids: co-chromatography with an authentic standard, UV/Vis spectrum, and mass spectrum that allows the

Table 5 Antioxidant capacity of pigment from *C. fucicola* 416

	µmol Trolox equivalent (TE)/mg of pigment					
	DPPH[c]	ABTS-H[d]	ABTS-L[e]	ORAC-H[f]	ORAC-L[g]	ORAC-CAT[h]
416	3.73 ± 0.11[a] (I% = 12.76)[b]	3.56 ± 0.67 (I% = 12.17)	3.82 ± 0,42 (I% = 19.23)	1.36 ± 0.03	5.12 ± 0.41	6.48

[a]Values expressed as the mean of triplicate ± standard deviation

[b]Percent inhibition % I

[c]DPPH, sequestration of the 2,2-diphenyl-1-picrylhydrazyl radical

[d]ABTS-H, capture of the 2,2-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS +) radical of the hydrophilic fraction

[e]ABTS-L, capture of the 2,2-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS +) radical of the lipophilic fraction

[f]ORAC-H, absorption capacity of the oxygen radical from hydrophilic fraction

[g]ORAC-L, absorption capacity of the oxygen radical from lipophilic fraction

[h]ORAC-CAT, absorption capacity of the total oxygen radical

identification of the molecular ion (Britton et al. 2004; Takaichi 2014). Moreover, working with carotenoids demands considerable precautions, for example, the complete execution of the analysis within the minimum possible time, exclusion of oxygen, protection from incident light, protection against high temperatures and acids, and use of high purity solvents (Rodriguez-Amaya and Kimura 2004; Rodriguez-Amaya 2016). Herein the three significant carotenoids identified from *C. fucicola* 416 are: zeaxanthin, β-cryptoxanthin and β-carotene. Two other minor carotenoids were identified as zeaxanthin isomers.

Zeaxanthin and β-cryptoxanthin are C40 xanthophylls and β-carotene is a C40 carotene (Fig. S2). They are naturally found in other bacteria (Kleinig et al. 1977; Takaichi et al. 1990; Yokoyama et al. 1996; Sajilata et al. 2008), including cyanobacteria (Hertzberg and Jensen 1966), and archaea (Kushwaha et al. 1972). These compounds are commercially exploited by the nutraceutical industry in different ways, such as cancer-preventive capabilities (Abahusain et al. 1999; Nishino et al. 2009), antioxidant functions (Fiedor and Burda 2014; Takahashi et al. 2016), obesity prevention (Burri 1997; Hammond et al. 2002; Burri et al. 2016), and even increased longevity (Cutler 1984). They are used in food products, vitamin supplements, cosmetics and health products (Del Campo et al. 2007). B-cryptoxanthin and β-carotene, as precursors of vitamin A, have been considered as protective agents against senile cataracts and aging macular degeneration (Olson 1989). In the food industry sector, the global trend for healthier foods began with the aim of producing natural colourants instead of artificial pigments (Delgado-Vargas et al. 2000). Natural carotenoids are a popular non-toxic additive to foods such as ice cream, butter and sweets. Zeaxanthin is already used for poultry and fish feed colourants (Nelis and De Leenheer 1991). In prokaryotes the carotenoids are located in membranes, mainly due to their lipophilicity, what makes them suitable photo-protectants and antioxidants (Gammone et al. 2015). They are probably involved in mediating phototaxis and phototropism (Nelis and De Leenheer 1991), ensuring protection against photodynamic killing (Mathews and Sistrom 1959; Mathews-Roth 1987; Carbonneau et al. 1989; Sajilata et al. 2008).

The carotenoids mixture of iridescent bacteria present higher antioxidant capacities in comparison to pigments produced by vegetables and other bacteria (Elisia et al. 2006; Tsai et al. 2007; Wang et al. 2010). Horta et al. (2014) described the antioxidant potential of the *Shewanella* sp. strain 16 pigments, which had an antioxidant protection effect of 3.603 μmol TE/g in the ORAC test, while the pigment from the polar bacterium strain 416 had effects of 6.480 μmol TE/g in the same test. The conjugated double bonds of carotenoids and the presence of functional groups results in their ability to quench singlet oxygen, which gives the antioxidant activity (Bohm et al. 2002; Britton et al. 2004; Fiedor and Burda 2014). Carotenoids are also efficient antioxidants protecting plants and contribute to the first and second defense lines against oxidative stress (Krinsky 2001; Stahl and Sies 2003).

The results obtained with carotenoids from *C. fucicola* 416 showed a significant potential source of chromophores to be used as UVA-filters in sunscreens; however, they were not considered photostable, which means that some compounds present in the extract degraded after irradiation while others remained stable. When submitted to the 3T3 NRU Phototoxicity test, the pigment extract presented a phototoxic potential when analysed in monolayers. This means that, in preliminary tests with cell cultured fibroblast, the mixed carotenoids were not considered safe for topic ingredient/formulation. However, the molecular weight of these carotenoids is higher than 500 Da, indicating that they would have low skin penetration in the viable layers of the human skin, which can lead to the absence of phototoxicity in vivo. To confirm this hypothesis a phototoxicity assay using a skin model would be necessary, allowing one to assess the bioavailability and penetration of these molecules on human skin. Since 2000, the in vitro 3T3 NRU phototoxicity test is the only in vitro test required for evaluating the acute phototoxicity of a chemical. However, this monolayer culture of fibroblasts is a simple basic system as compared to the three-dimensional architecture of skin involving connections and interactions between different cells types and the extracellular matrix (Augustin et al. 1997). Moreover, carotenoids in high concentrations become pro-oxidant as well (Palozza 1998; Young and Lowe 2001), which means that the monolayer assay developed in this study may have been conducted in a concentration range where the antioxidant properties had become pro-oxidant and for that reason damaged the cells.

Finally, for the development of new compounds to be used as UV filters or adjuvants for cosmetic formulations, subsequent studies with the isolated

molecules must be conducted to evaluate which carotenoids are responsible for the phototoxic activity and if this could be reduced with purification, to assure their photosafety.

The enormous economic potential of carotenoids is generally accepted for application in foods, feeds and cosmetics. Despite the current interest in 'natural' pigments from microorganisms, the production yield is still limited when compared with other cheaper sources of carotenoids from vegetables (Nelis and De Leenheer 1991; Taylor 1984). Thus, prospecting pigments in extremophilic bacteria opens different avenues in the search of carotenoids that are not found in plants, with further biotechnological applications that could result in a more sustainable and environmentally friendly way of exploiting natural resources. Moreover, the identification of pigments produced by the bacteria may help us understand ecological issues such as how bacteria thrive in the high UV and cold environments in the Antarctic continent.

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Author contribution RC-G and JE helped with the carotenoids identification. MVNR, FNdosS, MNE, helped with HPLC/MS interpretations. IAN-N and GMP performed the antioxidant tests. RSNT, HMD and LRGC performed the phototoxicity and photostability tests. LHR was the responsible for the Antarctica expedition, who made the sample collection feasible. VMO is the chief of laboratory.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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Supplementary Material (ESM)

Pigments in iridescent bacterium *Cellulophaga fucicola* isolated from Antarctica.

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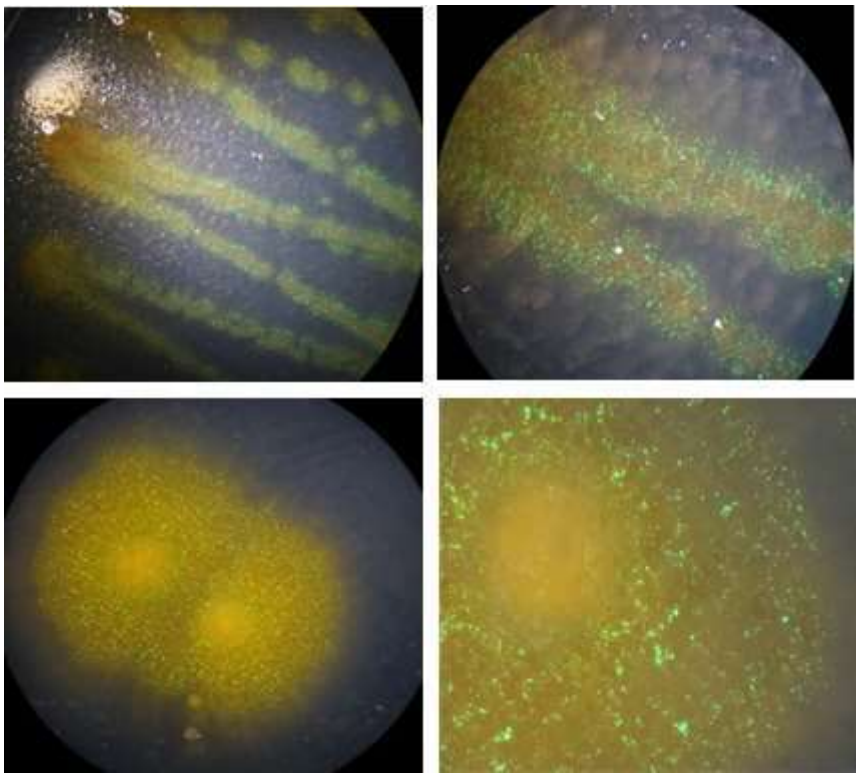
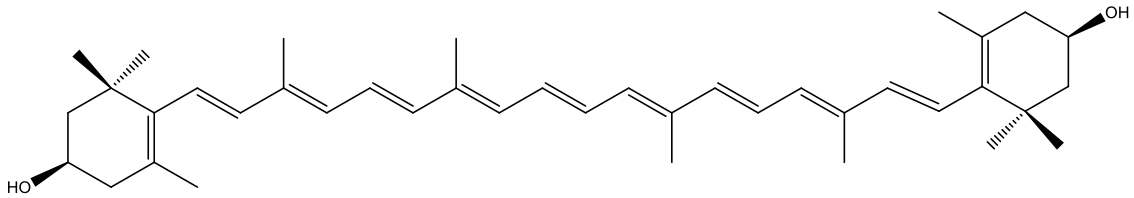
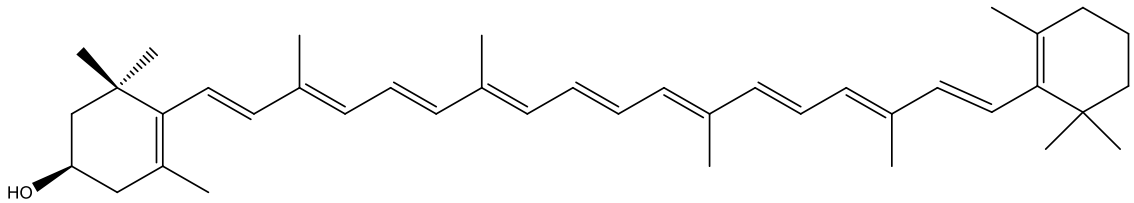


Fig S1. Macroscopic observation of *C. fucicola* 416 colony under oblique direct illumination.

Zeaxanthin



β –Cryptoxanthin



β –Carotene

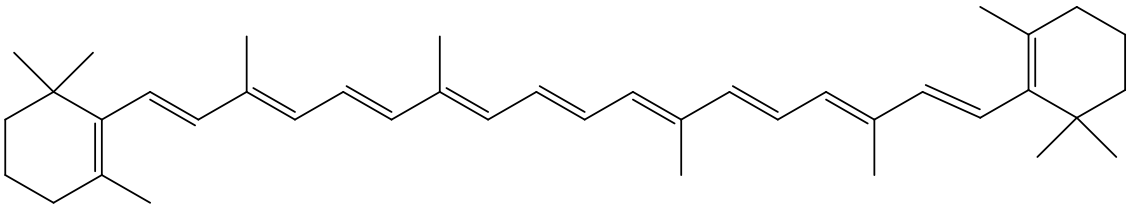


Fig. S2. Chemical structure of C40 carotenoids – zeaxanthin, β –cryptoxanthin and β –carotene isolated from *C. fucicola* 416.

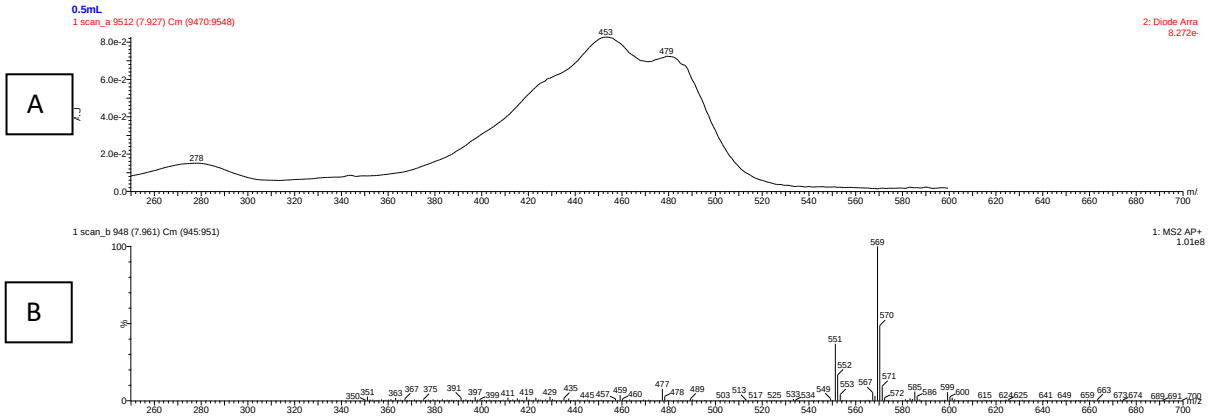


Fig. S3. Peak 7.96 min (A) UV/vis and (B) 300-700 *m/z* spectra. The protonated molecule at 569 and the fragmentation at 551 *m/z* correspondent to zeaxanthin.

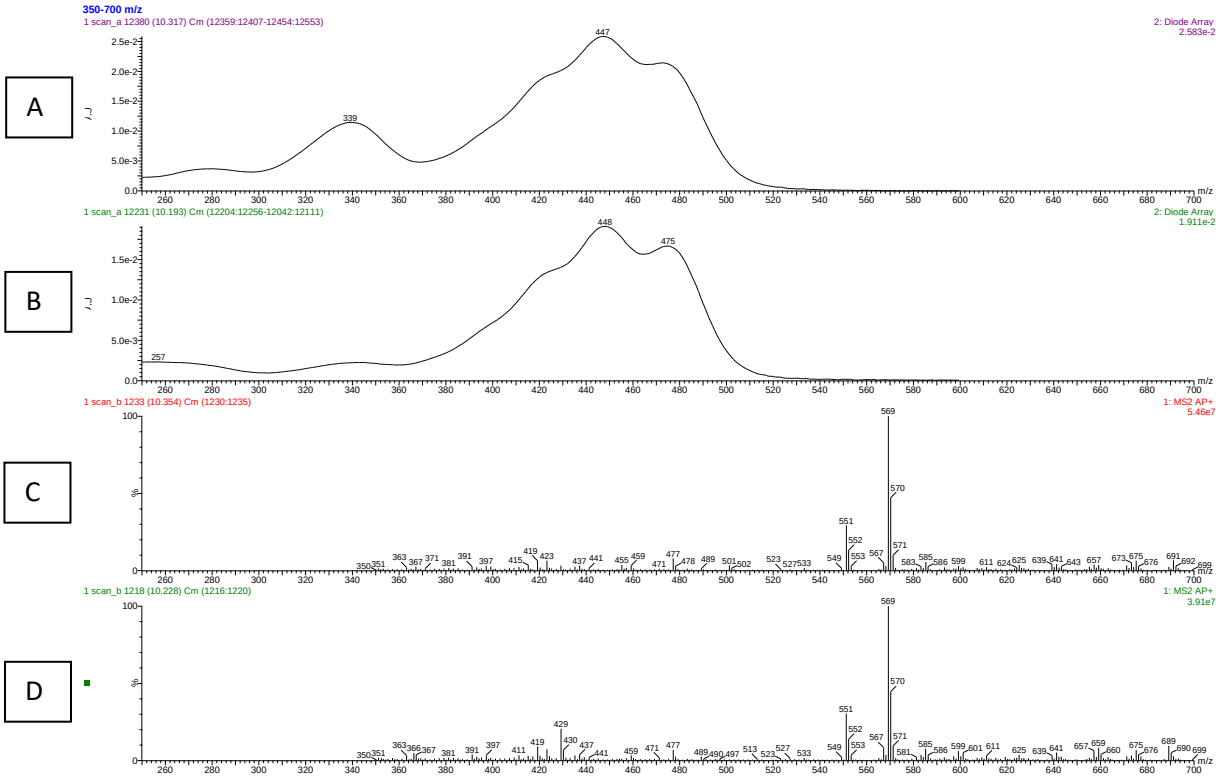


Fig. S4. Peak 10.19 min and 10.31 min (A) and (B) UV/vis respectively; (C) and (D) MS 300-700 *m/z* respectively spectra. The protonated molecule at 569 and the fragmentation at 551 *m/z* correspondent to zeaxanthin isomers.

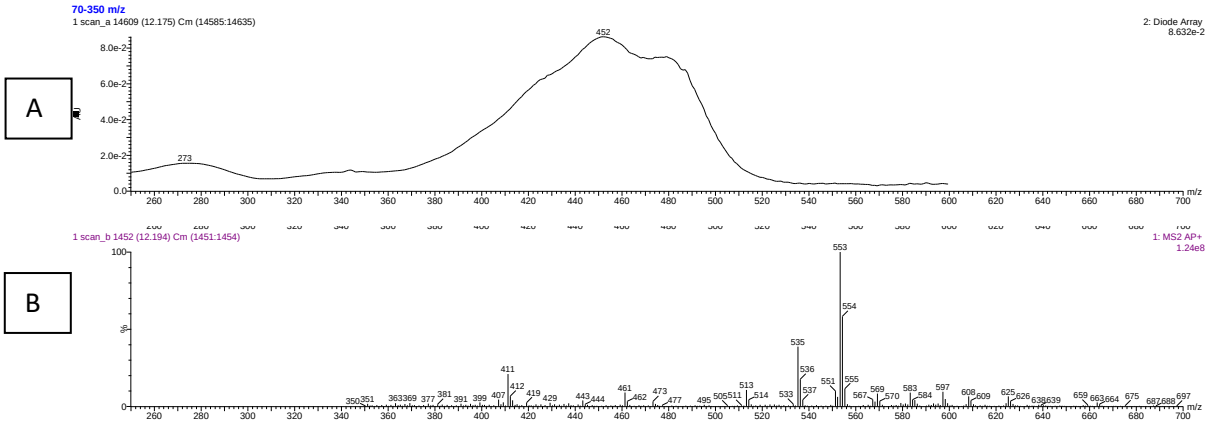


Fig. S5. Peak 12.17 min: (A) UV/vis and; (B) MS 300-700 *m/z* from peak 12.17. Identified as β-cryptoxanthin, protonated molecule *m/z* 553 and fragmentation at *m/z* 535.

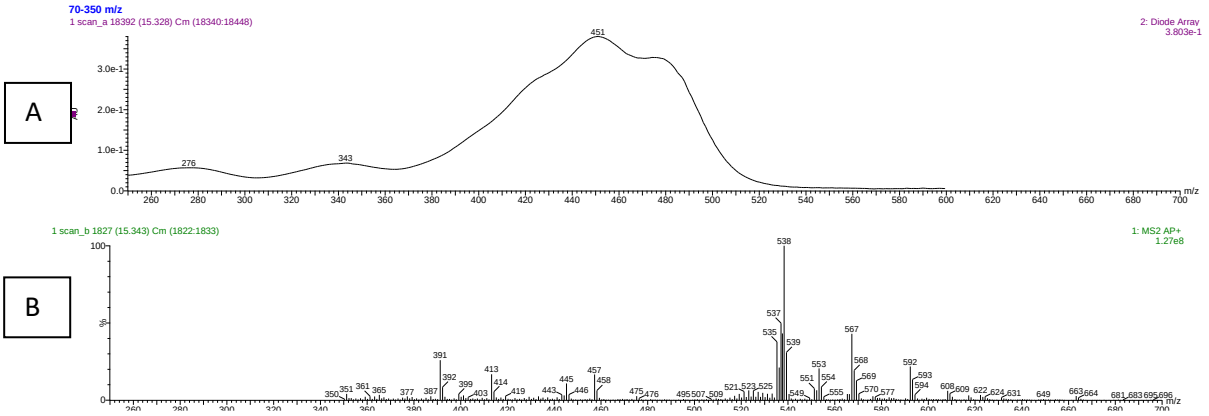


Fig. S6. Peak 15.3 min : (A) UV/vis; and (B) MS 300-700 *m/z* spectra from peaks 15.3. Identified as β-carotene, protonated molecule *m/z* 538.

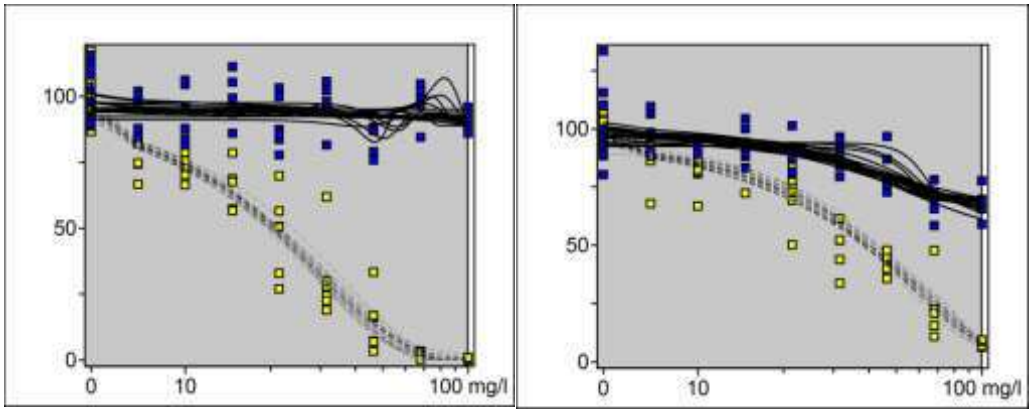


Fig. S7. The dose–response curves (duplicate) of pigment crude extract, obtained by the 3T3 NRU phototoxicity test and plotted using the Phototox 2.0 software program. The blue (solid lines) and yellow squares (dotted line) refer, respectively, to non-irradiated substances (-UV) and irradiated ones (+UV). Evaluated doses: 6.8, 10, 14.7, 21.4, 31.6, 46.4, 68.1 and 100 µg/mL.

Table S1. Photostability of *C. fucicola* 416 pigments.

	Maintenance of absorbance after irradiation		
	UVB	**UVA**	**Visible**
***C. fucicola* 416**	6,223/7,7895=79,9% 8,166/11,026=74,0% Mean= 76,5%	10,382/14,9605=69,4% 13,243/17,2945=76,6% Mean = 73%	47,6735/56,8465= 83,86% 41,7355/56,23= 74,21% Mean = 79%

DISCUSSÃO E PERSPECTIVAS FUTURAS

No presente estudo, 326 bactérias foram identificadas pelo sequenciamento do gene RNAr 16S e 45 delas podem representar espécies desconhecidas. Um estudo taxonômico mais profundo é necessário para caracterizar completamente os isolados que não foram agrupados com espécies já descritas nas árvores filogenéticas. Dessa forma, os resultados de diversidade revelaram um grande potencial de descoberta de novas espécies de bactérias entre os isolados da Antártica.

Os resultados do rastreamento de substâncias antimicrobianas, antiproliferativas e antiparasitárias demonstraram que há uma riqueza inexplorada nos ambientes antárticos para a bioprospecção de compostos com potencial de aplicação na indústria farmacêutico. No entanto, o pequeno número obtido de isolados produtores de antibióticos e a relativa baixa inibição das linhagens indicadoras corroboraram achados anteriores, sugerindo que as bactérias do frio da Antártica não são tão boas quanto seus parentes de ambientes mesofílicos para prospecção antimicrobiana. Uma bactéria, *Pseudomonas* sp. 99, demonstrou especial destaque nos testes de inibição e apresentou também resultados promissores nos ensaios antiproliferativos e antiparasitários. Trabalhos adicionais são necessários para elucidar a estrutura da molécula bioativa produzida por *Pseudomonas* sp. 99. Uma pista encontrada foi que essa bactéria afiliou-se proximamente à linhagem *Pseudomonas* sp. BTN1, isolada do mar Ross (Antártica), produtora de raminolipídios com atividade antimicrobiana (Tedesco et al., 2016), o que poderia guiar uma eventual identificação futura desse composto.

No entanto, respondendo à pergunta da introdução dessa tese, a buscar de novas moléculas antimicrobianas no continente Antártico, deveria ser tentada através de uma abordagem de "*high throughtput screening"* para maximizar as chances de se encontrar novos produtores. Aliado a isto, poderiam ser utilizadas ainda metodologias inovadoras de cultivo, como o iChip - dispositivo criado para acessar aquelas bactérias ditas não cultiváveis (Nichols et al. 2010) e outras variáveis possíveis, como a recuperação intra e extracelular dos extratos produzidos em diferentes condições de

crescimento, utilizando possíveis indutores de atividade antimicrobiana e testes contra um número maior de alvos (Nuñez-Monteiro et al. 2018).

No caso do recém-descoberto antibiótico *Teixobactina* (Ling et al. 2015), por exemplo, foram investigadas 10.000 bactérias isoladas em iChip, e uma delas - *Eleftheria terrae* - até então não cultivada, produziu um composto capaz de inibir o crescimento de *Staphylococcus* resistentes a meticilina. No ambiente Antártico, talvez um único estudo em larga escala tenha sido desenvolvido nesse sentido por Rojas et al. (2009), que resultou em 6348 extratos de 723 bactérias diferentes e chegou a elucidar um peptídeo de *Arthrobacter* sp. com potencial antimicrobiano. Para trabalhar com uma quantidade de extratos como esta, ambos os trabalhos citados usaram de tecnologias avançadas, principalmente a robótica, mecanização de processo e aparelhagem de química analítica refinada. No artigo de Spellberg et al. (2013), os autores citam que se fóssemos prospectar antibióticos em 10.000 actinomicetos diferentes de solo (um dos filos mais versáteis na produção de compostos antimicrobianos), 2.500 deles produziriam algum antibiótico. Desses, cerca de 2.250 seriam estreptotricinas, 125 estreptomicinas, e 40 tetraciclinas, e a vancomicina seria de se esperar encontrar uma em cem mil e a eritromicina seria esperado encontrar uma em um milhão de amostras triadas. Não que tenhamos exaurido as possibilidades de prospecção de novos antibióticos, muito pelo contrário, com as novas ferramentas cada vez mais avançadas, temos mais condições de acessar recursos metabólicos de micro-organismos do ambiente. Atualmente, no entanto, para chegarmos a uma molécula que seja boa o suficiente para entrar no mercado, teríamos de combinar esforços multidisciplinares com altos investimentos financeiros.

A microbiota da Antártica tem potencial para produção de novos antibióticos? Possivelmente sim. Poucas regiões da Antártica têm sido estudadas, e elas se concentram principalmente nas regiões litorâneas e insulares. As amostragens do interior do continente, abaixo da calota de gelo, são ainda virtualmente desconhecidas. Talvez a microbiota polar não seja boa produtora de antibióticos, mas os metabólitos produzidos por elas, quando encontrados, geralmente são novidades

para a comunidade científica. A baixa quantidade de antimicrobianos originários dessa microbiota nos faz levantar algumas hipóteses: será que sob o estresse da baixa temperatura, os micro-organismos investem sua energia mais para se multiplicar do que para a competição interespecífica – levando à baixa produção e secreção de antimicrobianos - já que a abundância de células é muito baixa e não levaria à necessidade de disputa por nutrientes? Ou mesmo a modulação da produção e secreção de substâncias antimicrobianas no frio seria prejudicada?...

Muitos autores têm descoberto grande produção de substâncias exopoliméricas e exopolissacarídeos em isolados da Antártica (Mancuso Nichols et al. 2004; Nichols et al. 2005a; Nichols et al. 2005b; Poli et al. 2007), que mostram indícios de que a seleção natural nesse ambiente de certa forma possa trabalhar mais para unirem as comunidades microbianas em biofilmes para resistirem às condições poliextremas do que para se repelirem competitivamente. Essas suposições e os aspectos ecológicos de comunidades dos micro-organismos do frio ainda são muito incipientes. A aplicação das técnicas de multiômicas nos sistemas antárticos fornecerá um importante entendimento da diversidade microbiana, da capacidade funcional do ecossistema, das novas moléculas antibióticas e seu papel na modulação dessas comunidades microbianas (Nuñez-Monteiro et al. 2018).

A identificação dos pigmentos produzidos pelas bactérias da Antártica indicou que a maioria era de carotenoides. Um carotenoide em específico denominado "*all-trans*-bacterioruberina" apresentou um potencial para uso como fator de proteção solar, podendo ser testado, por exemplo, em formulação de cosméticos. Como já existem pigmentos como a astaxantina, usada como suplemento de ração para salmões, os pigmentos bacterianos poderiam ser testados também para essa finalidade alimentícia. Assim, representam uma oportunidade para explorar pigmentos de origens naturais, os quais apresentam pouca toxicidade, não deixam resíduos, não apresentam riscos ambientais e são rapidamente decompostos no ambiente.

Numa busca específica por fatores de proteção solar naturais, especificamente daquelas moléculas que absorvem na UVA, cabe ressaltar que não são apenas os

pigmentos que podem conferir essa proteção. Na verdade, seriam moléculas incolores que absorvem a energia no comprimento de onda entre 320 e 400 nm, fora do espectro visível, como é o caso dos aminoácidos MAAs (Whitehead e Hedges 2005). Dessa forma, em uma futura prospecção de agentes fotoprotetores, novas abordargens devem ser levadas em conta para acessar essas moléculas “invisíveis” aos nossos olhos.

Outra bactéria, *Cellulophaga fucicola* 416, chamou a atenção devido ao seu brilho resultante do fenômeno da iridescência. Pela primeira vez foram analisados e identificados os pigmentos nessa bactéria, os quais incluíam três carotenóides: zeaxantina, β-criptoxantina e β-caroteno. Dois outros carotenoides menores foram identificados como isômeros da zeaxantina.

Assim, a prospecção de pigmentos em bactérias extremofílicas abre diferentes perspectivas na busca de carotenoides que não são encontrados em plantas, com outras aplicações biotecnológicas que podem resultar em uma maneira mais sustentável e ambientalmente correta de exploração dos recursos naturais. Além disso, a identificação de pigmentos produzidos pelas bactérias pode nos ajudar a entender questões ecológicas, como a proliferação de bactérias em ambientes com alta radiação UV e frio extremo, como no continente antártico.

CONCLUSÕES

- O levantamento taxonômico das bactérias isoladas de diferentes amostras da Antártica revelou uma grande diversidade bacteriana, que inclui os filos já descritos anteriormente por outros autores – Actinobacteria, Firmicutes, Proteobacteria e Bacteroidetes. No entanto, em níveis taxonômicos superiores existe um potencial de descoberta de novas espécies de bactérias entre os isolados da Antártica.
- Uma bactéria identificada como *Pseudomonas* sp. 99 destacou-se na produção de compostos antimicrobianos, antitumorais e antiparasitário. O isolado afiliou-se proximamente à linhagem BTN1 produtora de ramnolipídios com atividade antimicrobiana.
- As bactérias *A. agilis* 50cyt, *A. psychrochitiniphilus* 366, *C. fucicola* 416 e *Z. laminarie* 465 apresentaram taxa de sobrevivência contra a radiação UV maior que o controle negativo *E. coli.*
- Os pigmentos das bactérias selecionadas como as mais resistentes à UV foram identificados como carotenoides, tais como zeaxantina, β-criptoxantina, β-caroteno, fitoeno, decaprenoxantina e bacteriorruberina.
- Os resultados obtidos da identificação dos pigmentos podem nos ajudar a entender como as bactérias se desenvolvem nesses ambientes de alta radiação UV e frio.
- Pela primeira vez foram identificados os pigmentos de uma bactéria iridescente.

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